PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF TRANSGENIC RICE

OVER-EXPRESSING C$_4$ GENES FROM MAIZE AND THE DIVERSITY AND

PLASTICITY OF C$_4$ PHOTOSYNTHESIS IN *ELEOCHARIS* (CYPERACEAE)

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of LESLEY RYANN MURPHY find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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From a photosynthetic point of view, yield of some crops may be further improved by increasing the photosynthetic capacity of the source leaves and/or by increasing partitioning of photoassimilate to organs of economic importance. C$_4$ plants are able to overcome photorespiration, and thus photosynthesize more efficiently, through an additional photosynthetic pathway (C$_4$) and specialized leaf anatomy (Kranz) that work together as a "CO$_2$ pump" to supply Rubisco with enriched CO$_2$ edging out O$_2$ as a competitive substrate. This photosynthetic mechanism endows C$_4$ plants a selective advantage over C$_3$ plants especially in warmer climates and during water deficits: higher photosynthetic capacity, and higher water and nutrient use efficiency. The C$_4$ syndrome is characterized by high activities of C$_4$ enzymes and Kranz anatomy (mesophyll and bundle-sheath cells). Traditionally, Kranz anatomy, which allows for the spatial separation of the C$_4$ biochemical steps, was believed to be required for the C$_4$ CO$_2$ concentrating mechanism in terrestrial plants. However, it is known that C$_4$-like mechanisms can be induced, without the presence of Kranz anatomy, in two submersed

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aquatic species (*Hydrilla verticillata* and *Egeria densa*). Most recently, the terrestrial C_4 plants *Bienertia cycloptera*, *B. sinuspersici*, and *Suaeda aralocaspica* (Chenopodiaceae) from Central Asia have been found to lack Kranz anatomy and function with specialized intracellular compartmentation. Thus, by introducing the necessary genes of the C_4 pathway to C_3 plants, it may be possible to engineer a C_4-like mechanism and improve photosynthesis without a requirement for Kranz anatomy. In this study, C_3 rice was transformed with two genes encoding C_4 photosynthetic enzymes (phosphoenolpyruvate carboxylase and pyruvate orthophosphate dikinase) and the photosynthetic, biochemical, and anatomical changes were documented.

Additionally, research with the genus *Eleocharis* shows that the C_4 syndrome may be more plastic than formerly believed. Within the genus, species were shown to possess the full range from C_3 to intermediate to C_4. In a species dependant manner, plants changed photosynthetic types whether grown terrestrially or submerged with concomitant changes in the C_4 enzyme activities, carbon isotope composition and leaf anatomy.

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Dedication

To my son, BJ, for being my constant inspiration to succeed, not just in science, but in life. To my mother, Lea, for being a driving force in life and constant nagging. To my sister, Erika, for her unfailing support and friendship. And to all of my friends for keeping me sane.

CHAPTER ONE

Introduction

Increases in rice grain yield are of utmost importance to world health in the coming decades. In the 1960's, world famine was predicted and was averted due to the introduction of new rice lines that allowed for an increase in the harvest index (HI), which is a measure of the ratio of grain yield to total biomass. The new lines of rice had a HI of 0.5, up from 0.3, approximately tripling grain yield in some countries (Dingkuhn and Kropff, 1996). Looking ahead, the population predictions again call for a need for increases in plant production, but it appears that with current rice lines the need cannot be met with conventional plant breeding. Marker assisted selection and genetic manipulation of rice, and other crops, is being turned to for assistance in increasing yields and plant resistance to diseases and insects. Recently, scientists at the International Rice Research Institute (IRRI) proposed that the only means of increasing the productivity of rice to meet the increasing demands for food in Asia was to genetically modify rice to perform C_4 photosynthesis (Mitchell and Sheehy, 2006, Sheehy et al., 2000). Additionally, other research has shown that the only remaining factor which can be manipulated to increase yield is photosynthesis, and that increases in leaf photosynthesis will increase yield, if other factors are held constant (Long et al., 2006).

The C_3 pathway had been well characterized by Calvin, Benson, and Bassham in the early 1950's using *Chlorella* and it was considered a universal pathway. However, in 1965, Kortschak and Karpilov reported independently that the initial products of photosynthesis were malate and aspartate in sugarcane and maize, respectively. Hatch and Slack followed these reports by performing pulse-chase experiments and determining many of the enzymes (PEPC, PPDK, and NADP-MDH) and processes of the C_4 pathway in the 1960-70's.

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C$_4$ plants grow best in warm climates under high light and temperature conditions, with a few exceptions. Temperature is a critical factor in determining the geographical distribution of C$_4$ plants. They generally will not grow where the night temperatures are below 10-15°C (Sage et al., 1999). There have been 19 families of angiosperms (of the more than 400 families) identified as having C$_4$ species (Sage, 2004) (see Table 1). It appears that the C$_4$ syndrome has evolved many times randomly and independently during evolution of angiosperms with the primary driving force of reduced atmospheric CO$_2$ concentrations (Ehleringer et al., 1991). Ecologically speaking, 8 of the world's 10 worst weeds are C$_4$ plants (Holm, 1969).

Plants with the C$_4$ syndrome are able to overcome photorespiration, and thus photosynthesize more efficiently, through an additional photosynthetic pathway (C$_4$) and specialized leaf anatomy (Kranz) that work together as a "CO$_2$ pump" to supply Rubisco with enriched CO$_2$, edging out competition by O$_2$ and photorespiration. This photosynthetic mechanism endows C$_4$ plants a selective advantage over C$_3$ plants under warmer temperatures, and under water deficits resulting in high photosynthetic capacity and high efficiency of water and nitrogen use. High water use efficiency is due to the ability to reduce stomatal aperture and still maintain sufficient CO$_2$ concentrations within the leaf around Rubisco. The reduced amount of Rubisco needed for maximal CO$_2$ assimilation may enable investment in nitrogen elsewhere within the plant, with an increase in nitrogen use efficiency.

What it takes to be C$_4$

There are four characteristics or traits that are considered the absolute minimum that a plant must have to be considered C$_4$ through the dual cell-Kranz system (See Edwards et al., 2001). They are:

a) cell-specific amplification of enzymes of C$_4$ photosynthesis (i.e. phospho*enol*pyruvate carboxylase [PEPC] in mesophyll, and C$_4$ acid decarboxylases and Rubisco in bundle-sheath cells), with complementary adjustments of photosystem and electron transport activities;

b) novel cell-specific organelle metabolite transporters;

c) symplastic connections of the spatially separated sources and sinks of 4C-dicarboxylic acid transport metabolites; and

d) barriers to CO$_2$ diffusion between the site of CO$_2$ fixation by PEPCase in mesophyll cells and sites of CO$_2$ release and refixation by Rubisco in bundle-sheath cells.

C$_4$ subtypes

There are three recognized biochemical sub-types of the C$_4$ syndrome: NADP-ME, NAD-ME, and PEP-CK. All three types occur in family Poaceae (Edwards and Walker, 1983). They are defined by differences in the products and organelles used throughout the function of the C$_4$ cycle, the carboxylation, and decarboxylation phase. In the carboxylation phase of the pathway, all of the C$_4$ subtypes use phospho*enol*pyruvate carboxylase (PEPC) as the initial carboxylation enzyme in the cytosol of the mesophyll cells. The PEPC enzyme from maize is a homotetramer of 110 kDa subunits that is light activated/deactivated by phosphorylation on a serine by a kinase-phosphatase system.

PEPC is phosphorylated by PEPC kinase, which is also light dependent and thought to be influenced by cytosolic pH (Shenton et al., 2006). When PEPC is active, it binds metal^{2+} and reacts with phospho*enol*pyruvate (PEP) and bicarbonate (HCO_3^-) to form oxaloacetate (OAA) and inorganic phosphate (P_I) (Kanai and Edwards, 1999). The HCO_3^- is formed from atmospheric CO_2 by carbonic anhydrase (CA). Non-enzymatic equilibration from CO_2 to HCO_3^- would be rate limiting for the C_4 pathway. CA is found only in mesophyll cells as its presence in bundle-sheath cells would cause futile cycling as C_4 acid decarboxylases generate CO_2, and Rubisco only binds carbon in the CO_2 form (Kanai and Edwards, 1999). Also present in all the sub-types is NADP-malate dehydrogenase (NADP-MDH). NADP-MDH from maize is a homodimer with 43 kDa subunits. In the C_4 cycle, NADP-MDH catalyzes the conversion of OAA (the product from the PEPC reaction), NADPH and H^+ to form malate and $NADP^+$. Mg^{2+} must also be bound to NADP-MDH for enzyme function. NADP-MDH is activated by the ferredoxin-thioredoxin *m* system, which reduces a disulfide group on the enzyme. Additionally, enzyme activity is further increased by a high NADPH/ $NADP^+$ ratio in the mesophyll cell chloroplasts in light (by photosystem I action) (Kanai and Edwards, 1999). Alternatively, the OAA is transaminated by an aspartate aminotransferase to form aspartate. The diversity is the decarboxylation phase of the C_4 pathway will be discussed in the context of the specific subtype.

NADP-ME plants, such as maize, sugar cane, and sorghum, decarboxylate malate (from the NADP-MDH reaction) via NADP-ME in the bundle sheath cell chloroplasts. NADP-ME subtypes are primarily malate formers with operation of a malate-pyruvate shuttle. Malate is transported from the mesophyll cells to the bundle sheath cells via

plasmodesmata and then into the bundle sheath cell chloroplast. NADP-ME from sugarcane is a homotetramer with 62 kDa subunits at pH 8, or homodimer at pH 7. The homotetramer is active in the light by possible light/dark regulation by the ferredoxin-thioredoxin m system. The substrates of the reaction are malate and $NADP^+$ and the products are CO_2, pyruvate, NADPH and H^+. Mg^{2+} and Mn^{2+} are also required for enzyme function (Kanai and Edwards, 1999). Released CO_2 is then available for reaction with Rubisco in the C_3 cycle. Pyruvate is transported from the bundle sheath cell chloroplast to the mesophyll cell chloroplast where it reacts with pyruvate orthophosphate dikinase (PPDK) in an ATP- and Mg^{2+}- dependent manner to produce PEP. This reaction is reversible but forms PEP because of high activities of pyrophosphatase and adenylate kinase.

Pyruvate + Pi + ATP $\rightarrow$ PEP + PPi + AMP (PPDK)

AMP + ATP $\rightarrow$ 2 ADP (adenylate kinase)

PPi $\rightarrow$ 2 Pi (pyrophosphatase)

Total: Pyruvate + 2 ATP $\rightarrow$ PEP + 2 ADP + 2 Pi

PPDK from maize is a homotetramer with 94 kDa subunits and is light activated by a Pi dependent dephosphorylation of a threonine residue and inactivated by an ADP dependent phosphorylation of a threonine residue by a single, bifunctional protein (Chastain et al., 2002, Chastain et al., 2006, Edwards et al., 1985). Thus the cycle is complete with one molecule of CO_2 released to Rubisco and the use of two ATP in the C_4 cycle. In this sub-type, the bundle sheath cell chloroplasts are agranal and thus have very low photosystem II activity, O_2 evolution and linear electron flow. This reduces the competition between photosystem I and NADP-ME for $NADP^+$ in the bundle sheath cell

chloroplast. Rather, bundle sheath chloroplasts generate ATP via photosystem I dependent cyclic photophosphorylation to support the C_3 cycle.

NAD-ME type C_4 plants operate mainly an aspartate-alanine shuttle in the C_4 cycle. The OAA produced from PEPC in the mesophyll cells is transaminated mainly to aspartate by aspartate aminotransferase, which then diffuses through the plasmodesmata to the bundle sheath cell. Once in the bundle sheath cell mitochondria, the aspartate is transaminated back to OAA and converted to malate by NAD-MDH. The malate is then decarboxylated by NAD-ME in the mitochondria. NAD-ME from *Eleusine coracana* is a homooctamer with 63 kDa subunits. The same enzyme from *Amaranthus hypochondriacus* is a heterotetramer with two 65 kDa subunits (with catalytic sites) and two 60 kDa subunits. Malate and NAD^+ react with NAD-ME to form CO_2, pyruvate, NADH and H^+ in mitochondria. The CO_2 then diffuses from the mitochondria into the cytosol and then to the chloroplasts for use in the Calvin cycle. Mn^{2+} is required for enzyme function (Kanai and Edwards, 1999). The pyruvate is transaminated to alanine by alanine aminotransferase in a reaction that is linked to the production of OAA from aspartate by glutamate and α-ketoglutarate. The alanine then diffuses back to the mesophyll cell where it is transaminated back to pyruvate (again, a linked reaction) and the pyruvate is available for reaction with the mesophyll cell chloroplast enzyme PPDK for the regeneration of PEP. The expense of using the cycle is 2 ATP as in NADP-ME type species (while NAD and NADH are recycled in the bundle sheath cell mitochondria) per CO_2 released to Rubisco.

The PEP-CK type cycle is more complicated in that it utilizes both PEP-CK and NAD-ME. The OAA in the mesophyll cell is either transaminated to aspartate or

converted to malate by chloroplastic NADP-MDH. The aspartate diffuses through plasmodesmata to the bundle sheath cell cytosol, where it is transaminated back to OAA and decarboxylated by bundle sheath cell cytosolic PEP-CK. PEP-CK from five C_4 species are hexamers with MW ranges of 67-71 kDa subunits. OAA and ATP react via PEP-CK in a Mn^{2+}-dependent manner to form CO_2, PEP, and ADP. The PEP then diffuses to the mesophyll where it is used by PEPC. Alternatively, the malate diffuses to the bundle sheath cell mitochondria for decarboxylation by NAD-ME to form pyruvate, which is transaminated to alanine, diffuses to the mesophyll cell, transaminated back to pyruvate to regenerate PEP by PPDK. Some forms of PEP-CK appear to be light/dark regulated by phosphorylation/ dephosphorylation (Kanai and Edwards, 1999). Thus, the shuttle of malate through mitochondrial NAD-ME, besides generating CO_2, generates ATP, which is used to support the PEP-CK decarboxylation reaction. The CO_2 released by the two decarboxylases then diffuses into the bundle sheath cell chloroplasts (for use in the Calvin cycle). This C_4 system consumes NADH in the C_4 cycle through NAD-ME but has less expenditure of ATP through PPDK.

The C_4 syndrome is generally characterized by high activities of C_4 pathway enzymes and the presence of Kranz anatomy (mesophyll and bundle sheath cells). Traditionally, Kranz (which means wreath or halo in German and was first recognized by Haberlandt in 1892 in *Cyperus*) anatomy, which allows for the spatial separation of the C_4 biochemical steps, was believed to be required for the C_4 CO_2 concentrating mechanism in terrestrial plants.

Aquatic C$_4$ species

It has been shown that C$_4$-like mechanisms can be induced, without the presence of Kranz anatomy, in two submersed aquatic species (*Hydrilla verticillata* and *Egeria densa*) (Casati et al., 2000, Magnin et al., 1997). *Hydrilla verticillata*, a primitive aquatic angiosperm, can shift to C$_4$ photosynthesis under low CO$_2$ conditions. CO$_2$ is fixed by PEPC in the cytoplasm to form malate. The malate is then transported to the chloroplasts where it is decarboxylated by NADP-ME to provide CO$_2$ to Rubisco (Bowes and Salvucci, 1989, Reiskind et al., 1997). This cycle occurs through only one type of chloroplast.

Terrestrial C$_4$ without Kranz anatomy

More recently, the terrestrial C$_4$ plants *Bienertia cycloptera, B. sinuspersici*, and *Suaeda aralocaspica* (synonym: *Borszczowia aralocaspica*) (Chenopodiaceae) from Central Asia have also been found to lack Kranz anatomy and perform C$_4$ photosynthesis in a single chlorenchyma cell type by spatial separation of dimorphic chloroplasts and decarboxylation through mitochondrial NAD-malic enzyme (Chuong et al., 2006, Edwards et al. 2004, Freitag and Stichler, 2000, Voznesenskaya et al., 2001, Voznesenskaya et al., 2003). The proposed scheme of C$_4$ photosynthesis in these species is like that of the Kranz NAD-ME type species. *Suaeda aralocaspica* has cytoplasmic compartments at opposite ends of elongated chlorenchyma cells. *Binertia* species have a peripheral cytoplasmic compartment and a central cytoplasmic compartment which are connected by cytoplasmic channels (Edwards et al., 2004).

Benefits of C_4

The physiological results of the changes in anatomy and biochemistry of C_4 plants compared to C_3 are lower CO_2 compensation points, greater carboxylation efficiency, lack of inhibition of photosynthesis by oxygen, and higher photosynthetic rates at high temperatures, high light and drought conditions. These changes are due to the CO_2 concentrating mechanism, greater water use efficiency, the specificity of PEPC for HCO_3^-, and its lack of O_2 sensitivity. C_3 plants only have higher photosynthetic rates than C_4 plants at saturating, supra-atmospheric, CO_2 levels or at low temperatures.

Making C_3 into C_4

With respect to the increased efficiency of the C_4 pathway, attempts are being made to incorporate this pathway into C_3 plants. To be successful in general, the previously stated requirements of what it takes to be C_4 will need to be met, including spatial separation of trapping of atmospheric CO_2 by PEPC and effective donation of CO_2 from C_4 acids to Rubisco. Besides interest in genetically modifying some crops to perform C_4 photosynthesis, having an inducible C_4 system (e.g. under conditions where CO_2 is most limiting), could be advantageous. Current efforts to genetically engineer C_4 traits into C_3 plants are based on the single-celled system of *Hydrilla verticillata*, i.e. incorporation of C_4 enzymes without Kranz anatomy and without dimorphic chloroplasts. Using an *Agrobacterium*-based transformation system, three genes of the C_4 pathway have been transformed into rice, *Oryza sativa*. First, the intact maize PEPC gene (with introns, exons and promoter) was introduced which localizes to the cytosol of mesophyll cells in transgenic rice (Ku et al., 1999) (Figure 1). These rice transformants have been reported to have an increase in the activities of CA and Rubisco (Jiao et al., 2002).

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The second introduction was a cDNA encoding the maize chloroplast-form (as opposed to the cytosolic-form) of PPDK with the transit peptide sequence, which is required for localization of the protein to the chloroplast, using the rice *Cab* promoter. Lastly, the rice cDNA of C_3-specific NADP-ME using the rice *Cab* promoter was introduced. The protein products of these two transformants also localize to chloroplast, as determined by immunocytochemistry (Tsuchida et al., 2001). Conventional cross-hybridization was then utilized to produce a two-way hybrid of PEPC and PPDK (Fukayama et al., 2001). Although there is no evidence these transformants perform C_4 photosynthesis, some initial results have shown increased tolerance to high light and less photooxidative damage and increases in photosynthesis (CO_2 assimilation rate). This included increased photochemical and non-photochemical quenching and increased PS II efficiency (measured by fluorescence) when exposed to photo-oxidative conditions in four transgenic lines (PEPC, PPDK, NADP-ME, and PEPC/PPDK) compared to wild-type (Jiao et al., 2002, Murphy et al., 2001). Additionally (and perhaps more importantly), there was some evidence of increases in grain yield, generally through increased panicle number (without a change in weight per seed), in all four transgenic lines compared to wild-type (Murphy et al., 2001). In addition to rice, other species have been transformed with C_4 photosynthetic genes, such as *Arabidopsis*, tobacco and potato (Hausler et al., 2002, Leegood, 2002, Matsuoka et al., 2001). The enzyme activities for various C_4 transformants range from 0.5 to 110 fold increases depending on gene and construct used (Matsuoka et al., 2001). The physiological and biochemical affects of these genes varied. Maize PEPC, overexpressed in tobacco, resulted in significantly higher levels of malate, resulting in lower pH, but no change in photosynthesis (Leegood,

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2002). In potato transformed with PEPC from *Corynebacterium glutamicum*, there was

in increase in dark respiration and a decrease in the CO_2 compensation point (Leegood,

2002). This pattern of inconsistency in results can be seen with all of the C_4 genes that

have been transformed into C_3 plants (Raines, 2006). However, in each case, different

constructs, donor organisms, promoters and/or hosts have been used (Hausler et al., 2002,

Matsuoka et al., 2001, Raines, 2006). Additionally, there has been question about

whether the CO_2 released from the decarboxylation of malate would be lost to the

atmosphere, resulting in a futile cycle without a CO_2 concentrating mechanism (Hausler

et al., 2002, von Caemmerer, 2003). Based on a model of C_3 characteristics, it was

concluded that while an engineered single cell C_4 would not be an efficient CO_2

concentrating mechanism, it could have a benefit of overcoming diffusion limitations,

thus providing more CO_2 to Rubisco, especially in conditions that result in low

intercellular CO_2 conditions, such as drought (von Caemmerer, 2003). Lastly, it is clear

that inter- (in the case of Kranz system) and intra-cellular mechanisms of metabolite

transport are needed to facilitate the movement of substrates and products to make any

engineered C_4 pathway efficient (Suzuki et al., 2006).

Future of C_4 genetic research

The plasticity of a few C_4 plants, especially that of *Eleocharis* species, is drawn

on as evidence of the ability of plants to adapt to growth conditions and as a potential

future source for mechanisms that could enable C_3 plants to be engineered with the

anatomical benefits of Kranz anatomy. This is because some species of *Eleocharis* can

switch their anatomy and photosynthetic mechanism from C_3 to C_4 or C_3 -C_4 intermediate

depending upon whether they are grown submerged in water or terrestrially (Ueno et al.,

1989, Ueno and Wakayama, 2004). This change can also be induced with abscisic acid (Agarie et al., 2002, Ueno, 1998) and the changes may be triggered naturally by CO_2 concentrations. Diffusion of CO_2 in water is 10^4 lower than it is in air. Depending on pH, the majority of available carbon in the aquatic environment may be in the form of bicarbonate, which would favor C_4 photosynthesis if growth were limited by availability of CO_2. If genetic triggers can be determined, the potential to install this ability into C_3 plants may prove invaluable. In the four chapter of the thesis, anatomical, biochemical, and physiological changes were evaluated to determine the mode of photosynthesis in four *Eleocharis* species based on their growth environment.

Another proposed direction in C_4 research is the use of species from the genus *Cleome* in family Cleomaceae. Among the known occurrence of C_4 species, *Cleome* is most closely related to *Arabidopsis* and thus there are many genetic tools available for manipulation of the genome and trait analysis. *Cleome* also has the benefit of having a smaller genome than the current model systems (namely maize), a relatively shorter life span, small plant size, and there are C_3 and C_4 species within the genus (Brown et al., 2005, Voznesenskaya et al., 2007). Work with *Cleome*s and *Arabidopsis* may enable the anatomical switches to be elucidated and the transformation of C_3 to C_4 may be made more practical.

Individual contributions to this study

Besides my own efforts, this research was made possible by collaborators and contributions by the following individuals.

Chapter 2: Plant material was that of Ku et al., 1999 and Dr. Ku performed Western blots for PEPC.

Chapter 3: Plant material was that of Fukayama et al., 2001 and Dr. Dongha Cho of Kangwon University, Korea performed the crossing and screening of PC and PPDK lines to create CK lines.

Chapter 4: All plants were collected from the wild, grown initially in the greenhouse, identified, and initial genetic evaluations performed by Dr. Eric Roalson. Drs. Roalson, Ku and Lee performed carbon isotope determinations. Mr. Barroca measured all *Eleocharis* aquatic photosynthesis, performed terrestrial and aquatic microscopy (immunolocalization and anatomical), and helped with measuring enzyme activities as well as assisting in plant maintenance. Dr. Ku and Mr. Barroca performed the Western Blots for Rubisco and PEPC in *Eleocharis*.

Table legends:

Table I: List of families known to have C_4 species (adapted from Sage 2004).

Table 1

Monocots	Dicots	
Poaceae	Acanthaceae	Euphorbiaceae
Cyperaceae	Aizoaceae	Gisekiaceae
Hydrocharitaceae	Amaranthaceae	Molluginaceae
	Asteraceae	Nyctaginaceae
	Boraginaceae	Polygonaceae
	Cleomaceae	Portulaceae
	Caryophyllaceae	Scrophulariaceae
	Chenopodiaceae	Zygophyllaceae

*Cleomaceae has been substituted for Brassicaceae

Figure legends:

Figure 1. Schematic of maize C_4 phospho*enol*pyruvate carboxylase (PEPC) gene used for rice transformation. From Ku et al., 1999.

Figure 1.
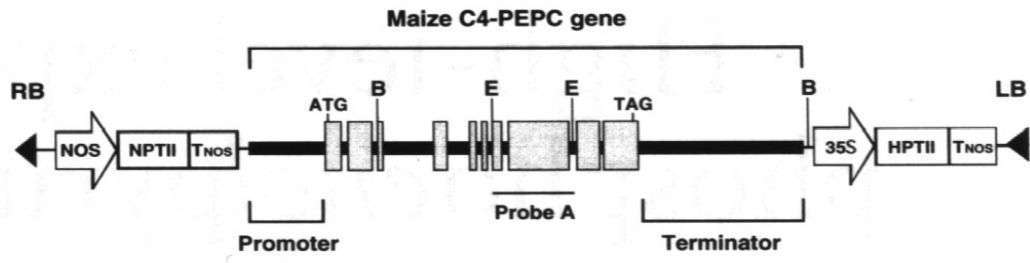
LITERATURE CITED

Agarie S, Kai M, Takatsuji H, Ueno O (2002) Environmental and hormonal regulation of gene expression of C_4 photosynthetic enzymes in the amphibious sedge *Eleocharis vivipara*. Plant Sci 163: 571-580

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. Aquat Bot 34: 233-286

Brown NJ, Parsley K, Hibberd JM (2005) The future of C_4 research-maize, *Flaveria* or *Cleome*? Trends Plant Sci 10(5): 215-221

Casati P, Lara MV, Andreo CS (2000) Induction of a C_4-like mechanism of CO_2 fixation in *Egeria densa*, a submersed aquatic species. Plant Physiol 123: 1611-1621

Chastain CJ, Fries JP, Vogel JA, Randklev CL, Vossen AP, Dittmer SK, Watkins EE, Fiedler LF, Wacker SA, Meinhover KC, Sarath G, Chollet R (2002) Pyruvate, orthophosphate dikinase in leaves and chloroplasts of C_3 plants undergoes light-/dark-induced reversible phosphorylation. Plant Physiol 128: 1368-1378

Chastain CJ, Heck JW, Colquhoun TA, Voge DG, Gu XY (2006) Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (*Oryza sativa*) seeds. Planta 224 (4): 924-934

Chuong SDX, Franceschi VR, Edwards GE (2006) The cytoskeleton maintains organelle partitioning required for single-cell C_4 photosynthesis in Chenopodiaceae species. Plant Cell 18: 2207-2223

Dingkuhn M, Kropff M (1996) Rice. In E Zamski, RA Schaffer, eds, Photoassimilate distribution in plants and crops. Source-sink relationships. Marcel Dekker Inc., New York, pp 519-547

Edwards GE, Walker DA (1983) 'C$_3$, C$_4$: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis.' (Blackwell Scientific Publications: Oxford, UK)

Edwards GE, Nakamoto H, Burnell JN, Hatch MD (1985) Pyruvate, Pi dikinase and NADP-malate dehydrogenase in C$_4$ photosynthesis: Properties and mechanism of light/dark regulation. Ann Rev Plant Physiol 36: 255-286

Edwards GE, Furbank RT, Hatch MD, Osmond CB (2001) What does it take to be C$_4$? Lessons from the evolution of C$_4$ photosynthesis. Plant Physiol 125: 46-49

Edwards GE, Franceschi VR, Voznesenskaya EV (2004) Single cell C$_4$ photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Bio 55, 173-196.

Ehleringer JR, Sage RF, Flanagan LB, Pearcy RW (1991). Climate change and the evolution of C$_4$ photosynthesis. Trends Ecol Evol 6: 95-99

Freitag H, Stichler W (2000) A remarkable new leaf type with unusual photosynthetic tissue in a central Asiatic genus of Chenopodiaceae. Plant Biol 2: 154-160

Hausler RE, Hirsch HJ, Kreuzaler F, Peterhansel C (2002) Overexpression of C$_4$-cycle enzymes in transgenic C$_3$ plants: a biotechnical approach to improve C$_3$ photosynthesis. J Exp Bot 53(369): 591-607

Holm L (1969) World's 10 worst weeds. Weed Sci 17: 113-118

Jiao D, Huang X, Li X, Chi W, Kuang T, Zhang Q, Ku MSB, Cho D (2002) Photosynthetic characteristics and tolerance to photo-oxidation of transgenic rice expressing C$_4$ photosynthetic enzymes. Photosynth Res 72: 85-93

Kanai R, Edwards GE (1999) The biochemistry of C_4 photosynthesis. In RF Sage, RK

 Monson, eds, *C_4 plant biology*, Academic Press, San Diego, pp. 49-88

Ku MSB, Agarie S, Nomura M, Fukayama H, Tsuchida H, Ono K, Hirose S, Toki S,

 Miyao M, Matsuoka M (1999) High-level expression of maize

 phospho*enol*pyruvate carboxylase in transgenic rice plants. Nat Biotechnol 17:

 76-80

Leegood RC (2002) C_4 photosynthesis: principles of CO_2 concentration and prospects for

 its introduction into C_3 plants. J Exp Bot 53 (369): 581-590

Long SP, Zhu XG, Naidu SL, Ort DR (2006) Can improvement in photosynthesis

 increase crop yields? Plant Cell Enviro 29: 315-330

Magnin NC, Cooley BA, Reiskind JB, Bowes G (1997) Regulation and localization of

 key enzymes during the induction of Kranz-less, C_4 -type photosynthesis in

 Hydrilla verticillata. Plant Physiol 115: 1681-1689

Matsuoka M, Furbank RT, Fukayama H, Miyao M (2001) Molecular engineering of C_4

 photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 52: 297-314

Mitchell PL, Sheehy JE (2006) Supercharging rice photosynthesis to increase yield. New

 Phytol 171: 688-693

Murphy LR, Huang XQ, Li X, Jiao DM, Cho D, Pinto M, Wong D, Li C, Yang J,

 Matsuoka M, Ku MSB (2001). Improved photosynthetic performance and grain

 yield in transgenic rice expressing maize C_4 photosynthesis enzymes. In

 Proceedings of the 12th International Congress on Photosynthesis.

Raines CA (2006) Transgenic approaches to manipulate the environmental responses of

 the C_3 carbon fixation cycle. Plant Cell Environ 29: 331-339

Reiskind JB, Madsen TV, Van Ginkel LC, Bowes G (1997) Evidence that inducible C_4-type photosynthesis is a chloroplastic CO_2-concentrating mechanism in *Hydrilla*, a submersed monocot. Plant Cell Environ 20: 211-220

Sage, RF, Li M, Monson RK (1999) The taxonomic distribution of C_4 photosynthesis. In: C_4 plant biology, RF Sage and RK Monson, eds, Academic Press: San Diego, pp. 551-584

Sage RF (2004) The evolution of C_4 photosynthesis. New Phytol 161: 341-370

Sheehy JE, Mitchell PL, Hardy B, eds. (2000) Redesigning rice photosynthesis to increase yield. Proceedings of the workshop on the quest to reduce hunger: Redesigning rice photosynthesis, 30 Nov-3 Dec. 1999, Los Baños, Philippines. Makati City (Philippines): International Rice Research Institute and Amsterdam (The Netherlands): Elsevier Science B.V. 293p.

Shenton M, Fontain V, Hartwell J, Marsh JT, Jenkins GI, Nimmo HG (2006) Distinct patterns of control and expression amongst members of the PEP carboxylase kinase gene family in C_4 family. Plant J 48(1): 45-53

Suzuki S, Murai N, Kasaoka K, Hiyoshi T, Imaseki H, Burnell JN, Arai M (2006) Carbon metabolism in transgenic rice plants that express phospho*enol*pyruvate carboxylase and/or phospho*enol*pyruvate carboxykinase. Plant Sci 170: 1010-1019.

Tsuchida H, Tamai T, Fukayama H, Agarie S, Nomura M, Onodera H, Ono K, Nishizawa Y, Lee BH, Hirose S, Toki S, Ku MSB, Matsuoka M, Miyao M (2001) High level expression of C_4 – specific NADP-malic enzyme in leaves and impairment of photoautotrophic growth in a C_3 plant, rice. Plant Cell Physiol. 42: 138-145

Ueno O, Samejima M (1989) Structural features of NAD-malic enzyme type C_4 *Eleocharis*: an additional report of C_4 acid decarboxylation types of the Cyperaceae. Bot Mag Tokyo 102: 393-402

Ueno O (1998) Induction of Kranz anatomy and C_4-like biochemical characteristics in a submerged amphibious plant by abscisic acid. Plant Cell 10: 571-583

Ueno O, Wakayama M (2004) Cellular expression of C_3 and C_4 photosynthetic enzymes in the amphibious sedge *Eleocharis retroflexa* ssp. *chaetaria*. J Plant Res 117: 433-441

von Caemmerer S (2003) C_4 photosynthesis in a single C_3 cell is theoretically inefficient but may ameliorate internal CO_2 diffusion limitations in C_3 leaves. Plant Cell Environ 26: 1191-1197

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414: 543-546

Voznesenskaya EV, Edwards GEE, Kiirats O, Artyusheva EG, Franceschi VR (2003) Development of biochemical specialization and organelle partitioning in the single-cell C_4 system in leaves of *Borszczowia aralocaspica* (Chenopodiaceae). Am J Bot 90(12): 1669-1680

Voznesenskaya EV, Noteyeva N, Chuong SDX, Ivanova A, Barroca J, Edwards GE (2007) Physiological, anatomical and biochemical characterization of photosynthetic types in genus *Cleome* (Cleomaceae). Func Plant Biol In press.

CHAPTER TWO

Physiological and biochemical analysis of transgenic rice over-expressing the

phospho*enol*pyruvate carboxylase gene from maize.

Abstract

In an effort to increase photosynthetic productivity and potential grain yield, *Oryza sativa* L. cv. Kitaake, rice is being transformed with genes of C_4 photosynthesis from maize (*Zea mays*). Rice (cv. Kitaake) transformed with the intact gene encoding the C_4 photosynthesis enzyme phospho*enol*pyruvate carboxylase (PEPC) from maize was used in the present study (line 81K-3). PEPC protein was localized to the mesophyll cytosol in high quantities with the highest levels of protein in the leaves and leaf sheaths. The transformed plants displayed increased photosynthetic rates under higher temperature, which was statistically significant at 40°C ($p=0.08$) and a reduced CO_2 compensation point ($p=0.08$). There was no significant change in Rubisco activity (per unit chlorophyll, leaf soluble protein, or leaf area) between the wild-type and transgenic rice. However, the transgenic rice had a slightly greater discrimination against $^{13}CO_2$ and a higher intercellular level of CO_2 suggestive of higher stomatal conductance. Leaf starch content appears to be lower in the transgenic PEPC rice, perhaps due to diversion of some products of CO_2 fixation by Rubisco to phospho*enol*pyruvate, the substrate for PEPC. The transgenic rice plants also had significantly more panicles per plant than the wild-type rice ($p=0.04$). The differences in results from various groups experimenting with the insertion of PEPC genes into C_3 plants may be due to several factors including selection of line from transformants, source of gene and construct used, or growth conditions.

<u>Introduction</u>

Photorespiration can account for up to a 50% decrease in photosynthetic capacity in C_3 plants, depending on environmental conditions, thus severely reducing their capacity for carbon assimilation (Edwards and Walker, 1983, Sage, 2000). The cause of photorespiration is the oxygenase activity of Rubisco and subsequent metabolism in the glycolate pathway. Approximately 6 to 8 million years ago, atmospheric CO_2 concentrations decreased to levels that were believed to cause up to 20% CO_2 loss due to photorespiration in C_3 plants (Sage, 2000). It is believed that it was at this time that C_4 photosynthesis became advantageous (Edwards et al., 2001). C_4 plants have an advantage over C_3 plants in conditions that would trigger excessive photorespiration (e.g. high temperature and water stress) because they possess a CO_2-concentrating mechanism that can maintain higher levels of CO_2 at the site of Rubisco. The C_4 syndrome is generally characterized by high activities of C_4 enzymes and the presence of Kranz anatomy (mesophyll and bundle sheath cells).

Phospho*enol*pyruvate carboxylase (PEPC), the initial carbon-fixing enzyme in the C_4 syndrome, is a homotetramer of 110 kDa subunits that is light activated by phosphorylation on a serine residue by a kinase-phosphatase system. When active, it binds metal^{2+} and reacts with phospho*enol*pyruvate (PEP) and bicarbonate (HCO_3^-) to form oxaloacetate (OAA) and inorganic phosphate (P_I) (Kanai and Edwards, 1999). This is a universal step in all C_4 plants. The OAA is then converted to malate by NADP-malate dehydrogenase or to aspartate by aspartate aminotransferase, which is then shuttled to the bundle sheath cells where the C_4 acids are decarboxylated. There are three C_4 subtypes based on three different C_4 acid decarboxylation enzymes, NAD-malic

enzyme, NADP-malic enzyme, and phospho*enol*pyruvate carboxykinase (Edwards et al., 2004). Traditionally, Kranz (which means wreath or halo in German and was first recognized by Haberlandt in 1892 in *Cyperus*) anatomy, which allows for the spatial separation of the C_4 biochemical steps, was believed to be required for the C_4 CO_2 concentrating mechanism. However, it has been shown that C_4-like mechanisms can be induced, without the presence of Kranz anatomy, in two submersed aquatic species (*Hydrilla verticillata* and *Egeria densa*) (Magnin et al., 1997; Casati et al., 2000). *Hydrilla verticillata*, a primitive aquatic angiosperm, can shift to C_4 photosynthesis under low CO_2 conditions. CO_2 is fixed by PEPC in the cytoplasm to form malate; the malate is then transported to the C_3 type chloroplasts where it is decarboxylated by NADP-ME (malic enzyme) to provide CO_2 to Rubisco (Bowes and Salvucci, 1989; Reiskind et al., 1997). More recently, terrestrial C_4 plants *Suaeda aralocaspica* and *Bienertia cycloptera* (Chenopodiaceae) from Central Asia have also been found to lack Kranz anatomy and perform C_4 photosynthesis in a single chlorenchyma cell type by spatial separation of dimorphic chloroplasts and decarboxylation through mitochondrial NAD-malic enzyme (Freitag and Stichler, 2000, Voznesenskaya et al., 2001, Voznesenskaya et al., 2003, Edwards et al. 2004).

Attempts at genetically engineering Rubisco to reduce the fixation of O_2 have not been successful; thus, our focus is to attempt to supplement C_3 plants with the biochemical pathways found in C_4 plants with the single-cell system as a model. Previous attempts to incorporate C_4 traits using breeding techniques were not successful (Ku et al., 1999). Using an *Agrobacterium*-based transformation system, three genes were introduced into rice, *Oryza sativa* cv. Kitaake (Ku et al., 1999). The genes encoding

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PEPC and PPDK (pyruvate orthophosphate dikinase) from maize, and rice NADP-ME were over-expressed. Through conventional hybridization a line that contains both PEPC and PPDK was developed. This chapter reports on the results with the PEPC line, hereafter referred to as PC.

Initial characterization of this line showed that the protein product encoded by the intact maize PEPC gene (with introns, exons and promoter) (Figure 1) localizes to mesophyll cells in leaves of transgenic rice and can account for up to 12% of total leaf protein (Ku et al., 2000). There was a concomitant increase in the activity of carbonic anhydrase, the enzyme that hydrates CO_2 to form bicarbonate, which is a substrate for PEPC (Ku et al., 2001, Jiao et al., 2002), while Rubisco activity in the PC transgenic lines was comparable to that of the wild-type (Jiao et al., 2002). It was also found, along with improved photosynthetic capacity, that the PC transgenic rice was better able to cope with oxidative stress (Ku et al., 2000, Jiao et al., 2002).

In this paper, we present data from a further series of biochemical and physiological studies to ascertain the effects of the insertion of the maize PC gene into rice.

<u>Results</u>

Diurnal enzyme activity

        The activity of PEPC in maize, wild-type and transgenic PC rice was followed from 6:45 am (before lights were turned on) to 4 pm (Figure 2). The diurnal activity of PEPC in typical C_4 plants shows an increase in activity during the first few hours of light exposure, which is expected, by the activative phosphorylation of the enzyme by phospho*enol*pyruvate carboxylase kinase (Vidal and Chollet, 1997). This pattern is seen in the maize control. However, the transgenic rice showed little or no activation. Wild-type rice shows very low levels of activity and very little activation. PEPC enzyme activity was significantly higher in the PC transgenic rice compared to wild-type at all time intervals (p=0.01, p<0.001, p=0.01, p=0.02, p=0.01, p=0.02, respectively).

Enzyme localization and activity

        Analysis of protein content via western blots and enzyme activity was made in different organs (leaf, sheath, stem, root and floret) from maize, wild-type rice and transgenic PC rice (Figure 3). In maize, the greatest enzyme content and activity is seen in the leaf and secondly the sheath (Figure 3A and B, respectively). In wild-type rice, PEPC levels are very low in all organs with the highest content in the stem and floret (Figure 3A). Transgenic PC rice shows high content and activity in the leaf and sheath, as does maize. In the root and floret the transgenic rice shows the same pattern as wild-type with comparable enzyme content (Figure 3A). There is a statistically significant difference in leaf PEPC enzyme activity between the PEPC transgenic rice and wild-type rice (p<0.001, Table 1).

Immunolocalization

Immunolocalization using the small subunit antibody to Rubisco shows it is localized in the chloroplasts of the mesophyll cells of both the wild-type (Figure 4A) and transgenic PC rice (Figure 4C).

Immunolocalization studies with the PEPC antibody shows the enzyme is localized in the cytosol of mesophyll cells in wild-type (Figure 4B) and transgenic PC rice (Figure 4D) with much heavier labeling in the transgenic plants.

Gas exchange

Several photosynthetic measures were made comparing wild-type and transgenic PC rice (Table I). The transgenic rice showed lower CO_2 compensation points than wild-type at both 21 and 2% O_2 (p=0.08) (Table 1 and Figure 5). The transgenic PC rice also showed on average slightly higher rates of assimilation at CO_2 saturation ($\sim$1400 μL L^{-1}) at both 2 and 21% O_2 although the increase was not statistically significant (Table 1 and Figure 5). Additionally, stomatal conductance was significantly higher in transgenic PC rice compared to wild-type rice at 2% O_2 (p=0.04), which is reflected in higher Ci/Ca ratios (Table 1). The carboxylation efficiency between the two plants was comparable at both 21% and 2% O_2 (Table 1 and Figure 6). Light compensation points, maximum incident quantum use efficiency, and light saturated assimilation rates were determined for the wild-type and transgenic PC plants at 21% O_2 and 360 μL L^{-1} CO_2 (Table I and Figure 7). While the wild-type and PC plants showed similar light compensation points (Table 1), the transgenic PC rice had generally higher quantum use efficiency, though not significantly different (Table 1 and Figure 7 inset) and on average higher maximum rates

of assimilation under saturating light and atmospheric levels of CO_2, but not significantly different (at 0.1 level) (Table I and Figure 7).

The optimum photosynthetic temperature for both the wild-type and transgenic PC rice was 35°C (Table I and Figure 8). However, the transgenic rice had higher rates of photosynthesis than the wild-type at both 35 and 40°C (significantly different at 40°C, $p=0.08$) (Figure 8 inset).

Rubisco activity and protein content

Rubisco activity was measured in leaf tissue of wild-type and PC transgenic rice on a per mg chlorophyll, mg protein, and area basis (Figure 9). There were no significant differences between the wild-type rice and the PC rice. There were also no significant differences between the wild-type and transformed rice in chlorophyll or soluble protein content.

Leaf ^{13}C content

Wild-type rice had a leaf δ^{13}C value of -26.3‰ while PC rice had a value slightly more negative, -27.0‰. While the difference between wild-type and transgenic PC rice is small (Table 1), it is significant ($p=0.05$).

Leaf starch content

Leaf cross-sections of wild-type (Figure 10A) and transgenic PC rice (Figure 10B) were stained using periodic acid plus Schiff's reagent to show starch content. All starch was located in the chloroplasts of the mesophyll cells. There appears to be lower starch content in the transgenic PC line compared to the wild-type.

Harvest data

Wild-type and transgenic PC rice was grown to maturity and harvested approximately 140 days after germination. There were significantly more panicles on the PC plants compared to the wild-type (p=0.04) and while there was no significant difference (p=0.23) in the grain yield (grams of grain/plant), the mean of the PC line was 30% higher than wild-type (Table 1).

Discussion

While it is apparent that there is much left to be done to create a "C_4 rice", this study indicates there are significant changes in the physiology of rice having high levels expression of only one of the C_4 genes from maize, PEPC.

The transgenic rice in this study (line 81K-3, generated in cv. Kitaake) had significantly higher activity of PEPC enzyme compared to wild-type rice. These plants also had statistically significantly lower CO_2 compensation points and $\delta^{13}C$ values and higher panicle numbers than the wild-type rice. These plants also had overall higher rates of photosynthesis with a statistically significant difference at 40°C. What is surprising is that there was no significant difference in Rubisco levels, since the overexpression of the PEPC enzyme would be thought to decrease the amount of protein allocated towards Rubisco. Additionally, while not statistically significant, the transgenic PEPC plants also, on average, had higher rates of photosynthesis under high light intensities, higher Ci/Ca ratios and 30% greater grain yield per plant.

The decrease in $\delta^{13}C$ and an increase in Ci/Ca points toward an increase in stomatal conductance resulting in greater CO_2 diffusion into the leaf, resulting in decreased diffusive resistance of gases allowing for higher internal carbon concentrations, thus increased discrimination by Rubisco and greater transpiration which allows for leaf cooling. This could assist the plants in their ability to have high photosynthetic rates at temperatures under which C_3 plants would normally be under high stress due to the decrease in CO_2 diffusion. The PEPC enzyme converts PEP and hydrated CO_2 to oxaloacetate, which can be reduced to malate by malate dehydrogenase. Malate in guard cells causes an influx of potassium ions and water, which causes stomata

to open. If malate levels are increased, this could explain an increase in stomatal conductance and an increase in Ci/Ca ratio. The stomatal conductance values were significantly higher in PC rice than wild-type at 2% O_2, but similar under 21% O_2.

Other theories for function of PEPC at the level of photosynthesis in the mesophyll cell could potentially increase photosynthesis and decrease the CO_2 compensation (but would increase the $\delta^{13}C$ values since PEPC does not discriminate). As noted by von Caemmerer (2003), if there were partial function of a C_4 cycle the diffusion resistance within the cells to CO_2 may be reduced, by the fixation of carbon by PEPC and subsequent decarboxylation, allowing Rubisco to work more efficiently. This would contribute to the lower CO_2 compensation point but would decrease CO_2 discrimination. PEPC could also lower the CO_2 compensation point by refixing photorespired CO_2 (Ku et al., 2001). While these are theories that need testing by more research, they may explain the increase in panicle number, as higher photosynthetic capacity will allow the plants to divert more carbon to reproduction.

Although PC rice does not have a conventional C_4 cycle, there may be other benefits, which, directly or indirectly, affect photosynthesis. Transgenic PC rice has been reported to have greater tolerance to photo-oxidative stress (Ji et al., 2004, Jiao et al., 2002, Jiao et al., 2005, Ling et al., 2006). Transgenic PC rice has been reported to have increased activity of the chloroplastic enzyme NADP-malate dehydrogenase (NADP-MDH) activity (Agarie et al., 2002, Rademacher et al., 2002). NADP-MDH is proposed to function to shuttle excess reductant out of the chloroplast under excess light through an oxaloacetate-malate shuttle (Scheibe, 2004).   Thus transgenic PEPC rice by producing increased levels of oxaloacetate in the cytosol may increase the capacity of the malate

shuttle, which could protect against photoinhibition by shuttling excess reductant from the chloroplast as well as potentially supplying malate to the mitochondria. Thus, higher rates of photosynthesis in PC rice compared to wild-type under high temperature and under high light may be due to protection against photoinhibition, which can occur under excess light.

Photosynthesis aside, there may be several metabolic changes in transgenic PC rice that would enable the plants to be more efficient and better able to cope with stress. As shown in Rademacher et al. (2002), elevated PEPC content caused increases in malate production in potato. If excreted from the roots, this would acidify the soils causing the release of otherwise unavailable nutrients, especially phosphorus (Schulze et al., 2002, Tesfaye et al., 2001; Tesfaye et al., 2003). Osaki and Shinano (2000) found that PC transgenic rice had improved tolerance to aluminum due to the release of oxalic acid from the roots. Similarly, over-expression of malate dehydrogenase, the enzyme that converts oxaloacetate to malate, in alfalfa improved aluminum tolerance through increased production of malate and other citric acids (Tesfaye et al., 2001).

There have been some conflicting results with PC rice transformants with use of the same gene construct from maize. Fukayama et al. (2003) reported higher photosynthetic rates for untransformed rice at both 21 and 2% O_2 and three different temperatures compared to their transgenic rice. As in the present study, they observed no change in Rubisco activity between transformed and untransformed rice; they attributed the reduction in photosynthetic efficiency by PC rice to increased respiration (Fukayama et al., 2003). However, the data presented here shows the transgenic PC rice on average had higher rates of photosynthesis at higher temperatures (35 and 40 °C), significantly

higher at the highest temperature tested (p=0.08), and at atmospheric oxygen concentrations under saturating light. There were also higher extrapolated respiration rates with transgenic PC rice, which showed a 16% (or 1.2 fold) increase in dark respiration compared to wild-type rice (extrapolated from data in inset of Figure 7).

Oxygen inhibition, calculated at saturated CO_2 concentrations (~1400 μL L^{-1}), was 12.5% for wild-type versus 6.8% in PC rice. At approximately current atmospheric CO_2 conditions, the O_2 inhibition values were similar (36.9 and 41.6%, respectively). However, at sub-atmospheric CO_2 levels (~80 μL L^{-1}), the PC rice had, again, a lower percent oxygen inhibition of photosynthesis (70.2%), compared to wild-type (79.4%) (calculated as in Agarie et al., 2002). Thus, the transgenic rice in our study had equivalent sensitivity to O_2 under atmospheric levels of CO_2, but on average lower sensitivity to O_2 at sub-atmospheric and super-atmospheric levels of CO_2 compared to wild-type (although not significant at $p = 0.49$ and 0.28, respectively). It has been suggested that lower sensitivity to O_2 in PC rice may be due to some limiting factor, such as Pi (Agarie et al., 2002) or Rubisco activity (Fukayama et al., 2003). However, in our study Rubisco levels were similar in wild-type and transgenic rice. Lower sensitivity to O_2 could also occur if there is increased conductance of CO_2 through stomata or if there is significant CO_2 fixation by PEPC under some conditions (since CO_2 fixation by PEPC is O_2 insensitive). In the latter case, PEPC may function as a secondary carboxylase, to synthesize C_4 acids, utilizing PGA derived from Rubisco.

Lastly, in the current study there were little, or no, diurnal changes in the activity of PEPC in the transgenic PC plants. In C_4 plants PEPC is activated in the light by the enzyme PEPC kinase. Thus, lack of light activation may be due to a deficiency of this

enzyme in the C_3 rice plant. These results are different from those of Fukayama et al. (2003) and Miyao and Fukayama (2003) who reported increased activity of PEPC in the dark in PC rice, and suggested this was analogous to CAM plants in which PEPC is activated in the dark through phosphorylation by PEPC kinase. Further studies will be needed to characterize the forms of PEPC kinase in rice plants to resolve these differences. The work of Matsuoka et al. (2000), Miyao (2003), Miyao and Fukayama (2003) and Fukayama et al. (2002, 2003) contradict other evidence suggesting beneficial effects on photosynthesis by Ku et al. (1999, 2001) and Jiao et al. (2002), as well as some data of Agarie et al. (2002), and data in the present study. Some of these differences were credited to results "unique to a particular transgenic rice plant" (Fukayama et al., 2003). The data presented in this paper are from the same line of plants that were used by Ku et al. (1999, 2001) and Jiao et al. (2002).

In summary, it is possible that line selection may play a part in the reported differences in performance between untransformed and transgenic PC rice, as well as growth and experimental conditions. Further analyses using different lines will be necessary to address the basis for these differences.

<u>Materials and Methods</u>

Plant material used

Transgenic PC rice used for all experiments in this paper were of the seventh generation, developed as reported in Ku et al., 1999, using a single parent of the line designated as 81K-3.

Plant growth conditions

All rice, *Oryza sativa* cv. Kitaake, used were grown in one-gallon pots, filled with a 4:1 mix of potting soil and clay, in growth chambers with a 12 hour photoperiod using a step-wise increase and decrease in light intensity with a maximum of 1000 μmol quanta $m^{-2} s^{-1}$ at the top of the canopy, a 26/20 °C day/ night temperature regime and 80% humidity. Plants were maintained with constant water supply and fertilized twice weekly with fertilizer (see following formulation) and once weekly with 50 mL of 10 mM KH_2PO_4, pH 7.4. All plants used for experiments were grown from seed from the same generation and collected from plants grown collectively to reduce any chance of variation. Plants were used approximately 6-7 weeks after germination except for data on reproductive development.

Rice Fertilizer formulation

Peters Hi Phos Special   15-30-15 - 2 parts

Calcium Nitrate   15.5-0-0 - 1 part

Peters All Purpose Fert. 20-20-20 - 1 part

Excel Magnitrate   10-0-0   - 1 part

Sprint 330 (Chelated Fe)   - 1/2 part

Peters S.T.E.M.   - 1/4 part (Soluble Trace Element Mix)

Enzyme assays and Western blot analysis

All enzyme assays were performed on crude extracts obtained by grinding leaf tissue harvested from the mid-portion of four to six newly mature leaves in the light in a cold (-80 °C) mortar and pestle with sterile acid-washed sand and a grinding medium containing 50 mM HEPES (or Tris) pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM Na-pyruvate, 10% glycerol, 5% insoluble-PVP, 5 mM iodoacetic acid, 10 mM $MgCl_2$, 1% β-mercaptoethanol. The extract was then centrifuged for 10 minutes at 14,000 x g at 4°C and the supernatant used for enzyme assay. Grinding media used for diurnal enzyme activity contained 0.1% Triton X-100.

For phospho*enol*pyruvate carboxylase (PEPC) assays, activity was measured spectrophotometrically at 340 nm by following the decrease in NADH in an assay solution containing 50 mM Tris-HCl pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.25 mM NADH, 1 mM G-6-P, 4 mM PEP, 5 mM DTT, and 12 units NAD-malate dehydrogenase at 30 °C.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was measured using a premix of 0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$, 50 mM DTT. Just before assaying 0.2 M $NaH^{14}CO_3$ and 20 uL extract were added and incubated at 30°C for approximately 2 minutes. The reaction was initiated by adding 15 μL of 5.0 mM RuBP and stopped after exactly 2 minutes by adding 50 μL of 1 N HCl/4 M Formic Acid solution. Samples were air dried, resuspended in 50 μL distilled deionized H_2O and 10 mL of scintillation cocktail and counted.

Westerns blots were performed as described in Ku et al. (1999).

Protein assays were done on supernatants using the Bio-Rad Assay kit (Hercules, CA) using BSA as the standard.

Leaf area measurements were made using a Licor LI-3000 portable leaf area meter (Lincoln, NE).

Chlorophyll extraction on intact leaves was done using ethanol by the method of Knudson et al. (1977), and by ethanol from leaf extracts obtained from grinding with mortar and pestle.

Immunolocalization

Immunolocalization was done by fixing leaf tissue samples in 1.25% glutaraldehyde/2% paraformaldehyde in 50 mM Pipes overnight at 4 °C, dehydrating in a series of alcohol dilutions and then embedded into LR White resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). Samples were cut at 100 nm using a Reichert R Ultracut (Reichert-Jung GmbH, Heidelberg, Germany), placed on slides and dried. The slides were blocked with TBST+BSA (10 mM Tris-HCl, 150 mM NaCl, 0.1% v/v Tween 20, 1% w/v bovine serum albumin, pH 7.2) for one hour and incubated in either maize anti-Rubisco (1:500 dilution) or anti-PEPC (1:1000 dilution) antibodies. After a series of rinses, they were incubated in 5 nm gold conjugated goat anti-rabbit antibody. After thorough rinsing, the slides were silver-enhanced per manufacturer's instructions (Amersham, Arlington Heights, Illinois, USA) and stained with 0.5% (w/v) Safrinin-O for 30 seconds. Confocal images were obtained on a Biorad 1024 MRC using the 20x objective at 2x electronic zoom and 10% laser power.

Gas exchange

Gas exchange measurements were obtained from three independent plants using two fully expanded leaves from plants that had not yet entered the reproductive stage using the FastEst gas system (FastEst, Tartu, Estonia); described in detail in Laisk and Oja, 1998) with a Licor LI-6251 infra-red gas analyzer (Lincoln, NE). Input gas humidity was adjusted for each temperature based on calculation of mV to relative humidity to maintain 80% humidity. Assimilation rates, determination of Ci, light intensity (PPFD) and leaf temperature were determined per Laisk and Loreto (1996).

Leaf ^{13}C content

Plant material, collected from mature plants, was dried at 60°C for 36-72 h then ground to a fine powder. Subsamples (1.5-2.0 mg) were analyzed by continuous flow isotope ratio mass spectrometry. A Eurovector elemental analyzer was used to combust samples and separate the resulting carbon dioxide gas, which was then analyzed for $^{13}C/^{12}C$ using a Micromass Isoprime mass spectrometer. Results are expressed as $\delta^{13}C = 1000 \times (R_{sample} - R_{standard})/(R_{standard})$, where $R_{standard}$ is Peedee belemnite (Craig, 1957). The precision based on replicate plant samples was generally in the range of 0.01-0.02 per mil.

Starch staining

Starch staining was done on LR White embedded leaf samples with periodic acid for one hour, rinsed and dried then stained with Schiff's reagent for one hour and dried. Images were obtained at 100x magnification using a light-microscope. Samples were collected simultaneously at mid-day.

Statistical analyses

All statistics were obtained with SigmaStat 2.03 using one-way ANOVAs, Tukey tests and Student t-tests. Transformations were employed to correct for heterogeneity when necessary. Comparisons were considered statistically significant for p values <0.1.

Table legends:

Table I: Comparison of physiological parameters between wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase (PC).   Results are average and standard error of three independent replicates. CO_2 compensation points, PEPC activity, leaf $\delta^{13}C$ and panicle number per plant are the only comparisons that were statistically significant. CO_2 compensation points were calculated from initial slopes of three separate experiments of A vs. Ci curves. Light compensation points were also calculated in this way from A vs. PPFD curves. Key: A=Assimilation rate ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$), Ci=intercellular CO_2, Ca=atmospheric CO_2, n.d.=no data.

Table 1

Parameter	wild-type		PC	
	21% O_2	2% O_2	21% O_2	2% O_2
CO_2 compensation point (μL L^{-1}) (p=0.08)	60.5±12.2	29.4±11.0	36.1±6.3	13.7±4.5
Carboxylation efficiency (mol m^{-2} s^{-1})	0.09±0.01	0.12±0.02	0.10±0.02	0.12±0.03
CO_2 saturated rate of A (μmol CO2 m^{-2} s^{-1})	53.6±2.1	61.7±4.1	60.7±4.9	65.1+/-6.5
Ci/Ca at max rates of A	0.56±0.16	0.65±0.16	0.81±0.19	0.82±0.17
Stomatal conductance at max rates of A (units) (p=0.04 at 2% O_2)	0.16±0.09	0.07±0.02	0.12±0.01	0.14±0.10
light compensation point (μmol quanta m^{-2} s^{-1})	42.3±3.8	n.d.	38.4±3.7	n.d.
Maximum incident quantum use efficiency	0.05±0.01	n.d.	0.06±0.001	n.d.
Light saturated rate of A (μmol CO_2 m^{-2} s^{-1})	25.8±4.8	n.d.	33.7±1.2	n.d.
Optimum photosynthetic temperature (°C)	35	n.d.	35	n.d.
Rubisco activity (μmol mg Chl $^{-1}$ min^{-1})	1.72±0.27	n.d.	2.16±0.17	n.d.
Rubisco activity (μmol mg protein^{-1} min^{-1})	0.06±0.0001	n.d.	0.06±0.01	n.d.
PEPC activity (p<0.001) (μmol mg protein^{-1} min^{-1})	0.05±0.002	n.d.	2.75±0.08	n.d.
Leaf $\delta ^{13}$C (‰) (p=0.05)	-26.3±0.2	n.d.	-27.0±0.1	n.d.
Panicle number per plant (p=0.04)	25±2.7	n.d.	34±4.6	n.d.
Grain yield (g per plant)	36.7±11.2	n.d.	47.8±7.3	n.d.

Figure legends:

Figure 1. Schematic of maize C_4 phospho*enol*pyruvate carboxylase (PEPC) gene used for rice transformation. From Ku et al., 1999.

Figure 2: Diurnal enzyme assay of phospho*enol*pyruvate carboxylase (PEPC) in *Zea mays* (Maize), wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize gene for PEPC (PC). Enzyme activity was followed over the course of the day with the lights turning on at 7:00 am. Data represents the mean and standard error of three independent replicates.

Figure 3: Comparison of phospho*enol*pyruvate carboxylase (PEPC) enzyme. A) protein amount via Western blot and B) activity in various organs of *Zea mays* (maize), wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize gene for PEPC (PC). L=leaf, S=sheath, ST=stem, R=root, F=floret. Data represents the mean and standard error of three independent replicates.

Figure 4: Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho*enol*pyruvate carboxylase (PEPC) immunolocalization in wild-type *Oryza sativa* cv. Kitaake (WT) A and B, respectively and transgenic rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase (PC) C and D, respectively. Confocal images were obtained using 20% laser power and 2x electronic magnification.

Figure 5: Oxygen sensitivity in wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic

rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase (PC). Gas

exchange conditions were 28°C, 80% relative humidity, 2000 μmol quanta m^{-2} s^{-1} at

either 21% O_2 or 2% O_2. Data represents the mean and standard error of three

independent replicates.

Figure 6: Carboxylation efficiency in A) wild-type *Oryza sativa* cv. Kitaake (WT) and

B) transgenic rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase

(PC). Gas exchange conditions were 2 and 21% O_2, 28°C, 80% relative humidity, and

2000 μmol quanta m^{-2} s^{-1}.

Figure 7: Light response curves from wild-type *Oryza sativa* cv. Kitaake (WT), and

transgenic rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase

(PC). Inset graph shows the initial slope of the curve used to determine quantum use

efficiency. Gas exchange conditions were 28°C, 80% relative humidity, 360 μL L^{-1} CO_2

and 21% O_2. Data represents the mean and standard error of three independent replicates.

Figure 8: Photosynthetic temperature response of wild-type *Oryza sativa* cv. Kitaake

(WT), and transgenic rice over-expressing the maize gene for phospho*enol*pyruvate

carboxylase (PC). Inset graph shows the percent increase in the assimilation rate of PC

over wild-type at each temperature. Gas exchange conditions were 360 μL L^{-1} CO_2, 80%

relative humidity, 1000 μmol quanta m^{-2} s^{-1} and 21% O_2. Data represents the mean and

standard error of three independent replicates. There are no statistically significant differences at $p < 0.1$ except for 40°C ($p = 0.08$).

Figure 9: A comparison of Rubisco activity on a chlorophyll, protein and leaf area basis and chlorophyll and soluble protein content in wild-type *Oryza sativa* cv. Kitaake (WT) and transgenic rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase (PC). The average and standard error of three independent replicates are shown. Values for μmol CO_2 fixed m^{-2} s^{-1} were divided by 10 and mg Chl cm^{-2} multiplied by 10 to fit scale.

Figure 10: Comparison of starch staining (PAS) in A) wild-type *Oryza sativa* cv. Kitaake (WT), and B) transgenic rice over-expressing the maize gene for phospho*enol*pyruvate carboxylase (PC).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Rubisco PEPC

WT

PC
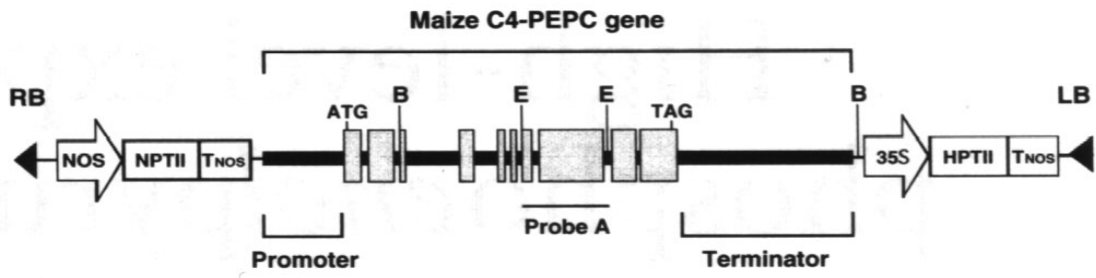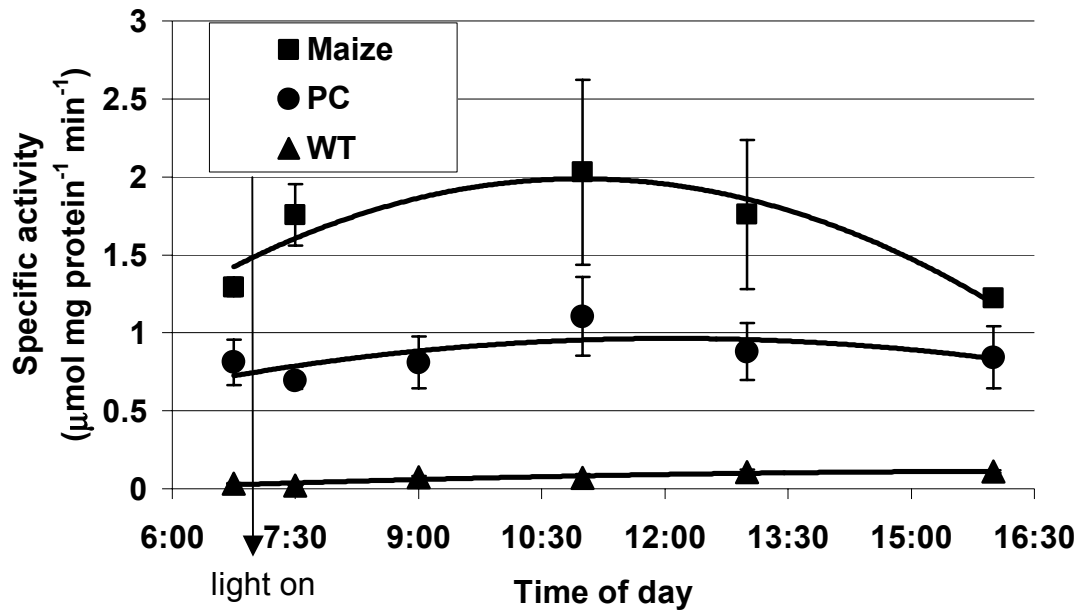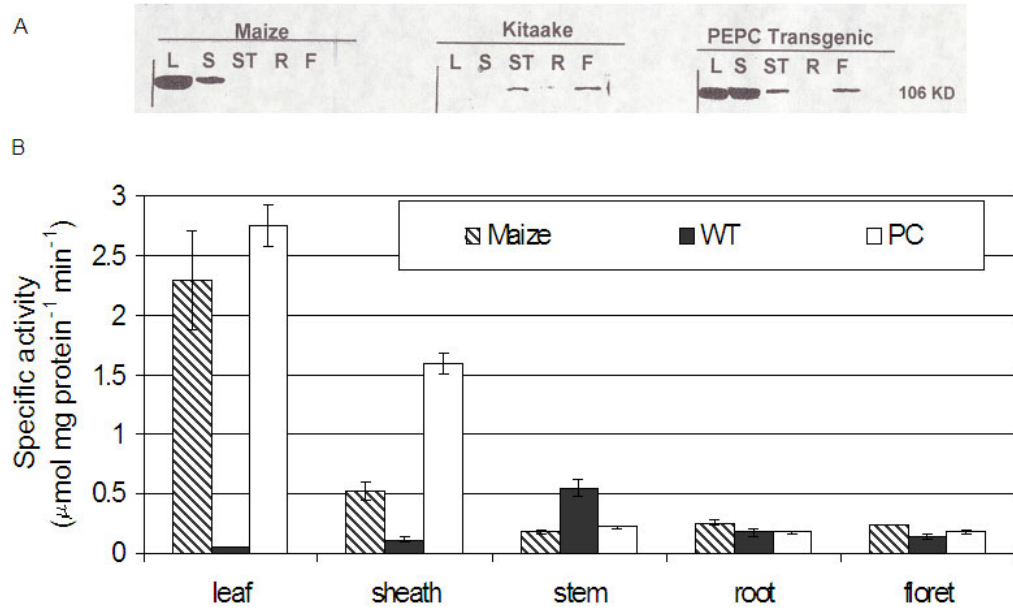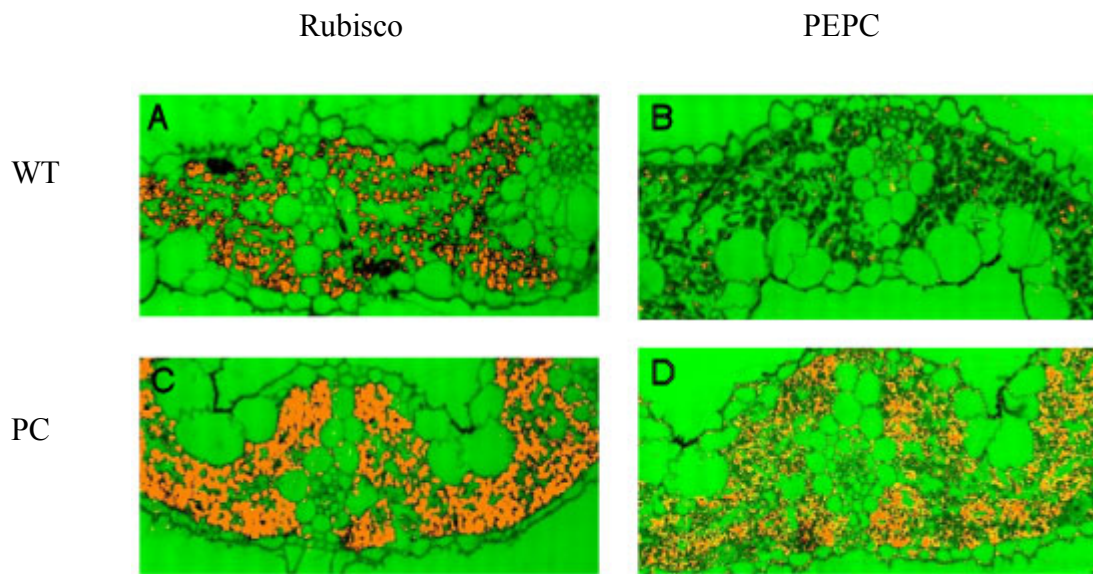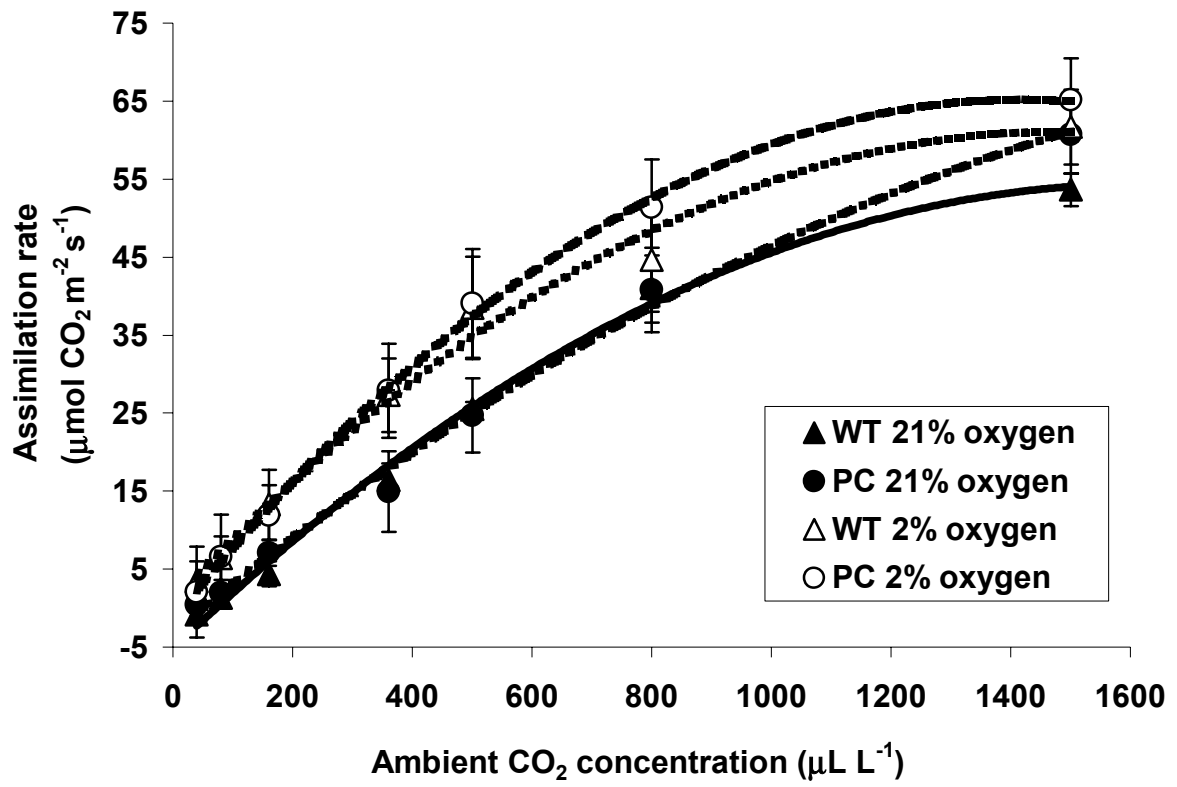
Figure 5.

Figure 6.

A
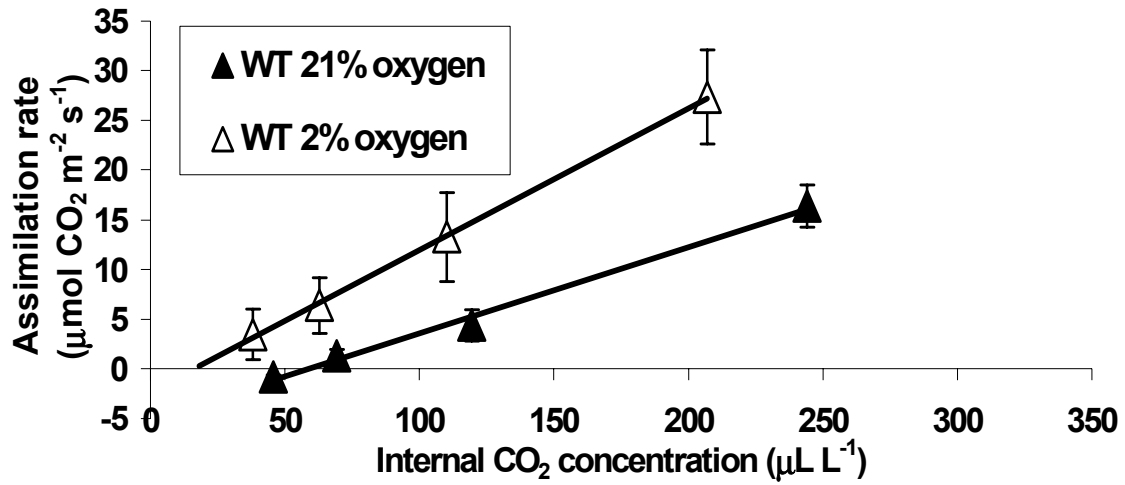
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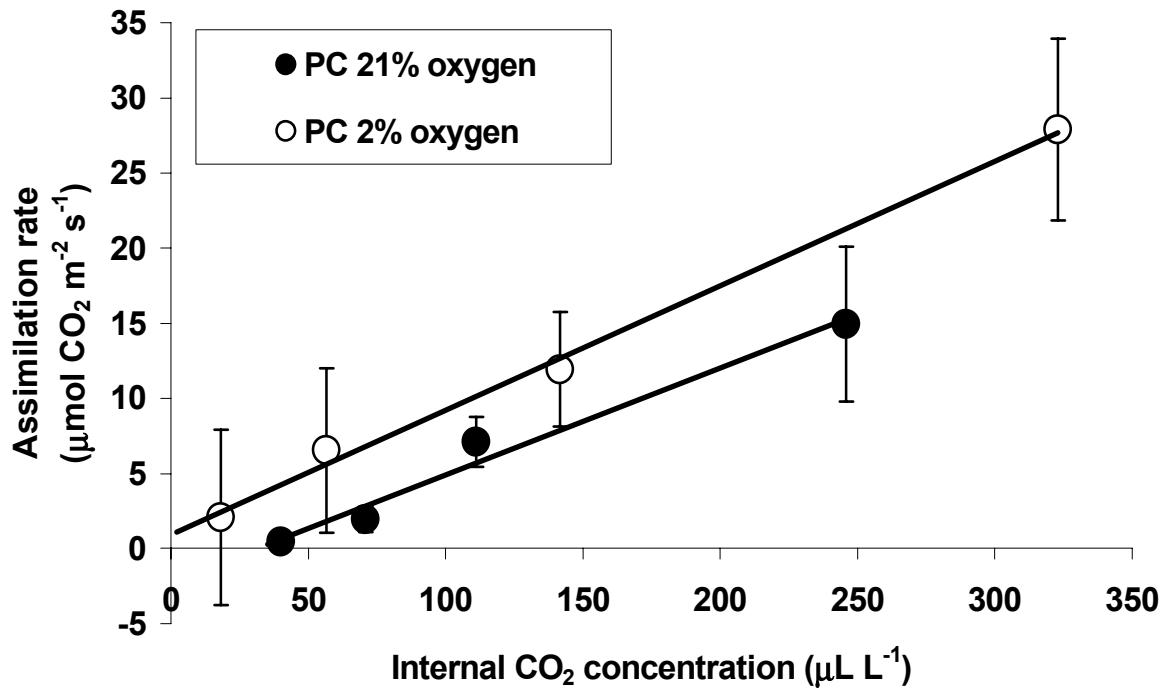
Figure 7.

Figure 8.

Figure 9.

Legend:
- $\mu mol\ CO_2\ mg\ Chl^{-1}\ min^{-1}$
- $\mu mol\ CO_2\ mg\ protein^{-1}\ min^{-1}$
- $(\mu mol\ CO_2\ m^{-2}\ s^{-1})/10$
- $mg\ protein\ cm^{-2}$
- $mg\ Chl\ cm^{-2}\ *10$
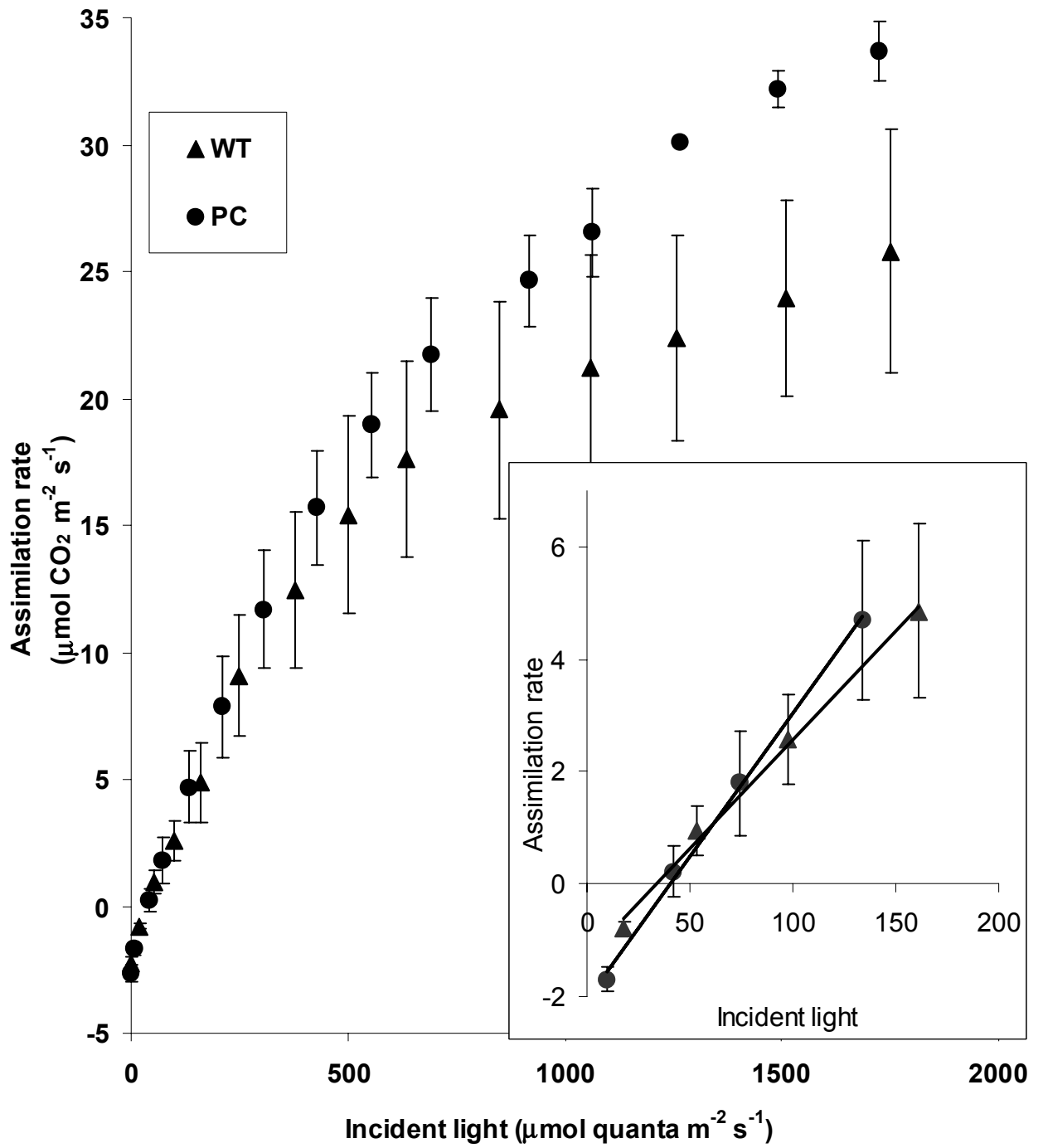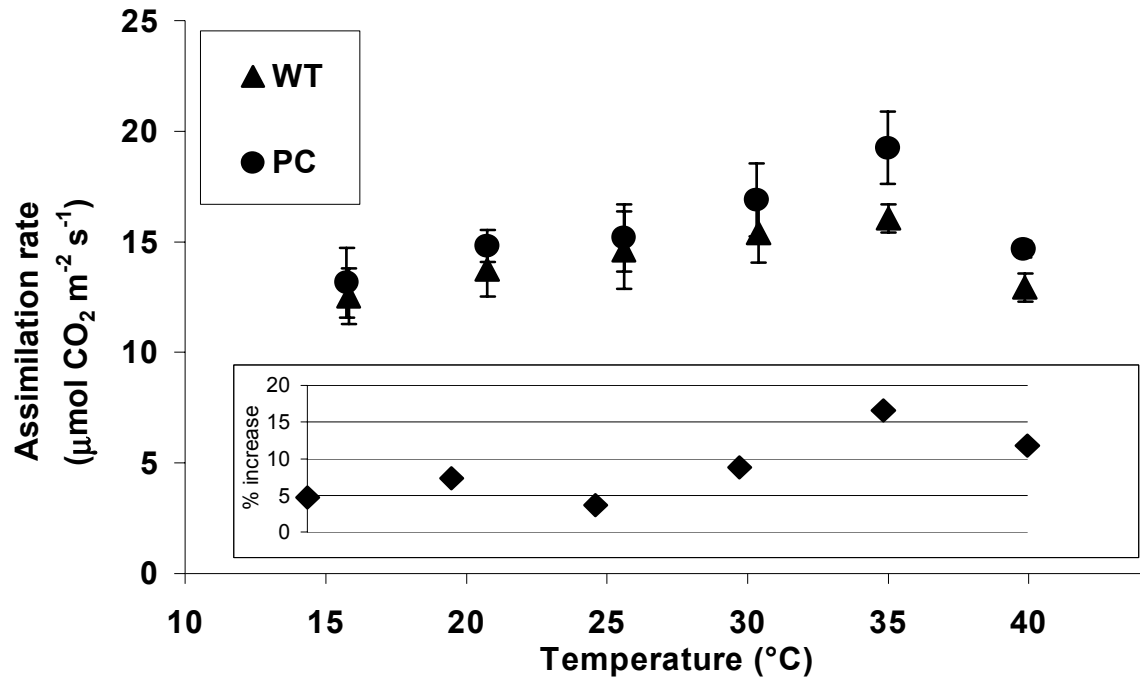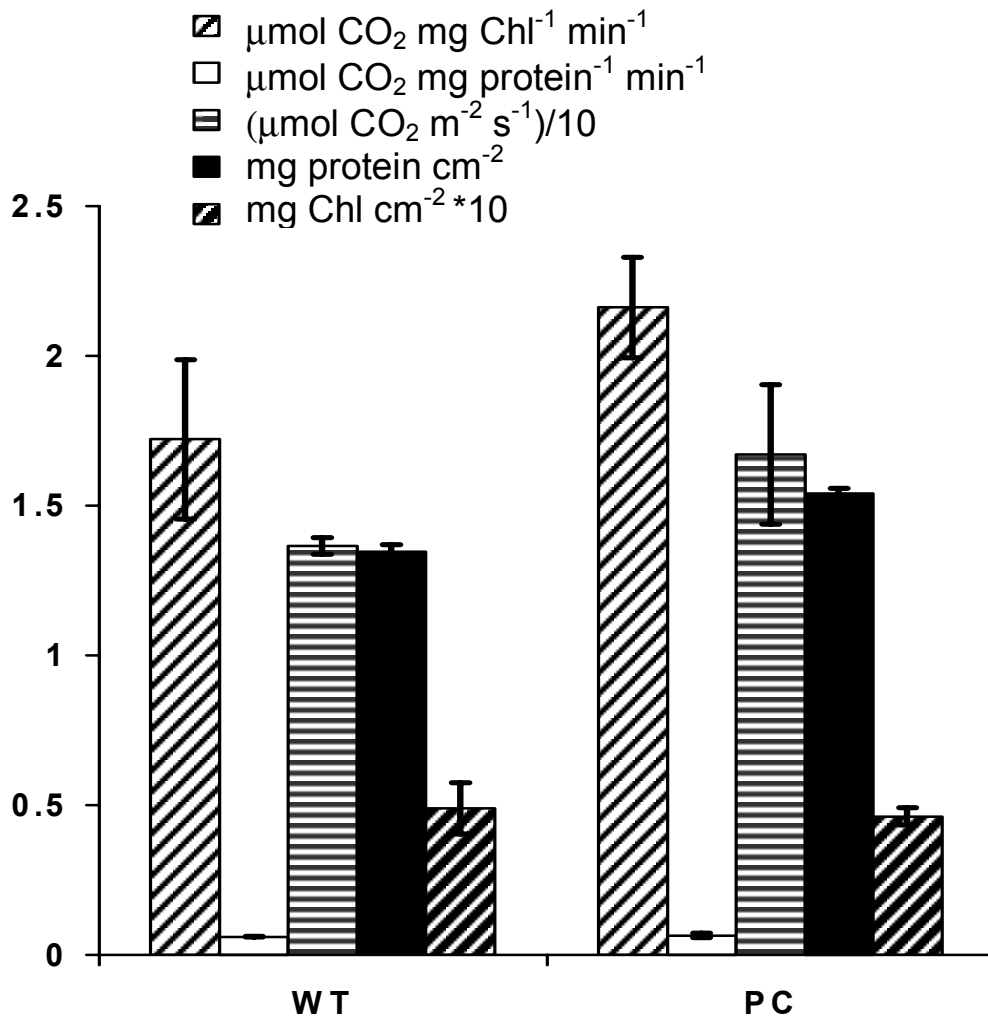
Figure 10.

A

B

LITERATURE CITED

Agarie S, Miura A, Sumikura R, Tsukamoto S, Nose A, Arima S, Matsuoka M, Miyao-Tokutomi M (2002) Overexpression of C4 PEPC caused O_2-insensitive photosynthesis in transgenic rice plants. Plant Sci 162: 257-265

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. Aquat Bot 34: 233-286

Casati P, Lara MV, Andreo CS (2000) Induction of a C_4-like mechanism of CO_2 fixation in *Egeria densa*, a submersed aquatic species. Plant Physiol 123: 1611-1621

Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. Geochim Cosmochim Acta 12: 133-149

Edwards GE, Walker DA (1983) 'C$_3$, C$_4$: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis.' (Blackwell Scientific Publications: Oxford, UK)

Edwards GE, Franceschi VR, Voznesenskaya EV (2004) Single cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Bio 55, 173-196.

Freitag H, Stichler W (2000) A remarkable new leaf type with unusual photosynthetic tissue in a central Asiatic genus of Chenopodiaceae. Plant Biol 2: 154-160

Fukayama H, Tamai T, Tsuchida H, Miyao-Tokutomi M (2002) Overproduction of the maize C4-specific PEPC enhances the respiration under illumination in transgenic rice plants. Plant Cell Physiol 43: S173

Fukayama H, Hatch MD, Tamai T, Tsuchida H, Sudoh S, Furbank RT, Miyao M (2003) Activity regulation and physiological impacts of maize C_4–specific phospho*enol*pyruvate carboxylase overproduced in transgenic rice plants. Photosynth Res 77: 227-239

Ji BH, Zhu SQ, Jiao DM (2004) A limited photosynthetic C_4 microcycle and its physiological function in transgenic rice plant expressing the maize PEPC gene. Acta Bot Sin 46(5): 542-551

Jiao DM, Huang X, Li X, Chi W, Kuang T, Zhang Q, Ku MSB, Cho D (2002) Photosynthetic characteristics and tolerance to photo-oxidation of transgenic rice expressing C_4 photosynthetic enzymes. Photosynth Res 72: 85-93

Jiao DM, Li X, Ji BH (2005) Photoprotective effects of high level expression of C_4 phospho*enol*pyruvate carboxylase in transgenic rice during photoinhibition. Photosynthetica 43(4): 501-508

Kanai R, Edwards GE (1999) The biochemistry of C_4 photosynthesis. In RF Sage, RK Monson, eds, *C_4 plant biology*, Academic Press, San Diego, pp.49-88

Knudson LL, Tibbitts TW, Edwards GE (1977) Measurement of ozone injury by determination of leaf chlorophyll concentration. Plant Physiol 60: 606-608

Ku MSB, Agarie S, Nomura M, Fukayama H, Tsuchida H, Ono K, Hirose S, Toki S, Miyao M, Matsuoka M (1999) High-level expression of maize phospho*enol*pyruvate carboxylase in transgenic rice plants. Nat Biotechnol 17: 76-80

Ku MSB, Cho D, Ranade U, Hsu TP, Li X, Jiao DM, Ehleringer J, Miyao M, Matsuoka M (2000) Photosynthetic performance of transgenic rice plants overexpressing maize C_4 photosynthesis enzymes. In JE Sheehy, PL Mitchell, B Hardy, eds, Redesigning rice photosynthesis to increase yield. Proceedings of the workshop on the quest to reduce hunger: redesigning rice photosynthesis. Elsevier Science B.V., IRRI and Amsterdam, pp. 193-204

Ku MSB, Cho D, Li X, Jiao DM, Pinto M, Miyao M, Matsuoka M (2001) Introduction of
genes encoding C4 photosynthesis enzymes into rice plants: physiological
consequences. In Rice biotechnology: improving yield, stress tolerance and grain
quality. Wiley, Chichester (Novartis Foundation Symposium 236) pp. 100-116

Laisk A, Loreto F (1996) Determining photosynthetic parameters from culm CO_2
exchange and chlorophyll fluorescence. Plant Physiol 110: 903-912

Laisk A, Oja V (1998) Dynamics of culm photosynthesis. Commonwealth Scientific and
Industrial Organization Publishing, Collingwood, Australia

Ling LL, Lin HH, Ji BJ, Jiao DM (2006) CO_2 exchange and chlorophyll fluorescence of
phospho*enol*pyruvate carboxylase transgenic rice pollen lines. J Integr Plant Biol
48(12): 1431-1438

Magnin NC, Cooley BA, Reiskind JB, Bowes G (1997) Regulation and localization of
key enzymes during the induction of Kranz-less, C_4 -type photosynthesis in
Hydrilla verticillata. Plant Physiol 115: 1681-1689

Matsuoka M, Fukayama H, Tsuchida H, Nomura M, Agarie S, Ku MSB, Miyao M
(2000) How to express some C_4 photosynthesis genes at high levels in rice. In JE
Sheehy, PL Mitchell, B Hardy, eds, Redesigning rice photosynthesis to increase
yield. Proceedings of the workshop on the quest to reduce hunger: redesigning
rice photosynthesis. Elsevier Science B.V., IRRI and Amsterdam, pp 167-175

Miyao M (2003) Molecular evolution and genetic engineering of C_4 photosynthesis
enzymes. J Exp Bot 54(381): 179-189

Miyao M, Fukayama H (2003) Metabolic consequences of overproduction of phospho*enol*pyruvate carboxylase in C_3 plants. Arch Biochem Biophys 414: 197-203

Osaki M, Shinano T (2000) Influence of carbon-nitrogen balance on productivity of C_3 plants and effect of high expression of phophoenolpyruvate carboxylase in transgenic rice. In JE Sheehy, PL Mitchell, B Hardy, eds, Redesigning rice photosynthesis to increase yield. Proceedings of the workshop on the quest to reduce hunger: redesigning rice photosynthesis. Elsevier Science B.V., IRRI and Amsterdam, pp 177-192

Rademacher T, Hausler RE, Hirsch HJ, Zhang L, Lipka V, Weier D, Kreuzaler F, Peterhansel C (2002) An engineered phosphoenolpyruvate carboxylase redirects carbon and nitrogen flow in transgenic potato plants. Plant J 32: 25-39

Reiskind JB, Madsen TV, Van Ginkel LC, Bowes G (1997) Evidence that inducible C_4-type photosynthesis is a chloroplastic CO_2-concentrating mechanism in *Hydrilla*, a submersed monocot. Plant Cell Environ 20: 211-220

Sage RF (2000) C_3 versus C_4 photosynthesis in rice: ecophysiological perspectives. In JE Sheehy, PL Mitchell, B Hardy, eds, Redesigning rice photosynthesis to increase yield. Proceedings of the workshop on the quest to reduce hunger: redesigning rice photosynthesis. Elsevier Science B.V., IRRI and Amsterdam, pp 13-38.

Scheibe R (2004) Malate valves to balance cellular energy supply. Physiol Plant 120: 21-26

Schulze J, Tesfaye M, Litjens RHMG, Bucciarelli B, Trepp G, Miller S, Samac D, Allan D, Vance CP (2002) Malate plays a central role in plant nutrition. Plant Soil 247: 133-139

Tesfaye M, Temple SJ, Allan DL, Vance CP, Samac DA (2001) Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. Plant Physiol 127: 1836-1844

Tesfaye M, Dufault NS, Dornbusch MR, Allan DL, Vance CP, Samac DA (2003) Influence of enhanced malate dehydrogenase expression by alfalfa on diversity of rhizobacteria and soil nutrient availability. Soil Biol and Biochem 35: 1103-1113

Vidal J, Chollet R (1997) Regulatory phosphorylation of C_4 PEP carboxylase. Trends Plant Sci 2(6): 230-237

von Caemmerer S (2003) C_4 photosynthesis in a single C_3 cell is theoretically inefficient but may ameliorate internal CO_2 diffusion limitations in C_3 leaves. Plant Cell Environ 26: 1191-1197

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414: 543-546

Voznesenskaya EV, Edwards GEE, Kiirats O, Artyusheva EG, Franceschi VR (2003) Development of biochemical specialization and organelle partitioning in the single-cell C_4 system in leaves of *Borszczowia aralocaspica* (Chenopodiaceae). Am J Bot 90(12): 1669-1680

CHAPTER THREE

Physiological and biochemical analysis of transgenic rice over-expressing the phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase genes from maize.

Abstract

Physiological, anatomical and biochemical studies were made on features of photosynthesis in previously developed homologous lines of rice having maize genes for phospho*enol*pyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK). C_3 rice, *Oryza sativa* L. cv. Kitaake, was transformed independently with maize genes encoding PEPC and PPDK. The intact gene for PEPC and the cDNA for PPDK with the rice *Cab* promoter were inserted using *Agrobacterium*-mediated transformation. The resulting plants were then crossed to develop a homozygous line with both genes (CK, line C4-32-5). The CK plants had a lower CO_2 compensation point at 2 and 21% O_2 compared to wild-type, which is expected if PEPC contributes to CO_2 fixation under limiting CO_2. CK plants also had a significantly lower light compensation point ($p=0.06$), suggestive of refixation of respired CO_2. There was also evidence of higher photosynthetic rates over a range of temperatures (15-30ºC) when compared to wild-type rice. The CK transgenic rice also had significantly more panicles per plant ($p=0.05$) and while not statistically significant, they had on average over 30% more grain yield per plant than wild-type. The insertion of these genes, while only part of the C_4 photosynthetic pathway in maize, has a measurable effect on rice photosynthesis and productivity, but whether it will be possible to create a functional C_4 cycle in rice remains to be seen.

<u>Introduction</u>

Rice (*Oryza sativa* L.) is an important staple crop to the majority of the world's population and with the dependence on this crop and the projected increases in world population in the coming decades, increases in crop yield are necessary. Using *Agrobacterium*-mediated introduction, C_4 photosynthetic genes have been introduced into some C_3 crops, such as rice, tobacco and potato, in attempt to increase photosynthetic capacity and crop yield (Hausler et al., 2002, Leegood, 2002, Matsuoka et al., 2001). C_4 photosynthetic characteristics are favorable due to their lack of photorespiration, which can account for up to a 50% loss in fixed carbon in C_3 plants under certain conditions (Hausler et al., 2002), as well as their greater water and nitrogen use efficiency (Sage and Pearcy, 1987). While attempts have been made to genetically manipulate Rubisco, to alleviate its oxygen fixing property, as well as the enzymes involved in the photorespiratory pathway to increase photosynthetic efficiency in C_3 plants, none of those approaches to date have been successful (Raines, 2006). A number of genes encoding the C_4 photosynthetic enzymes have been cloned and successfully introduced by several groups, however, none of the genes encoding the anatomical specialization of Kranz anatomy have been discovered. Thus, the single-cell C_4 model found in *Hydrilla verticillata* (Bowes and Salvucci, 1989), which uses phospho*enol*pyruvate carboxylase (PEPC) as its primary carboxylation enzyme, NADP-malic enzyme (NADP-ME) as its decarboxylation enzyme to release CO_2 for secondary fixation by Rubisco, and regeneration of PEP by pyruvate orthophosphate dikinase (PPDK), has been used. Futile cycling of CO_2 by PEPC and Rubisco due to lack of spatial separation and cell leakiness to CO_2 diffusion have been proposed deleterious consequences to using this model (von

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Caemmerer, 2003). However, *Hydrilla*, as well as other plants discovered since, successfully concentrates CO_2 without the cellular specialization as is seen in traditional Kranz C_4 plants (Edwards et al., 2004, Raines, 2006).

The transgenic rice used in this study was developed through traditional crossing of two transgenic rice lines that were independently homozygous for the insertion of maize C_4 genes encoding PEPC and PPDK, respectively, and then selfed and screened for homozygous lines for both genes. The intact PEPC gene and the cDNA for PPDK with the *Cab* promoter were used to produce the parental lines (Fukayama et al., 2001, Ku et al., 1999). Initial screening of the resultant transgenic plant (designated as CK) showed these plants to have higher photosynthetic rates and over 20% greater grain yield than untransformed rice in a naturally illuminated nethouse study by Jiao et al., 2002.

<u>Results</u>

Diurnal enzyme activity

The activity of PEPC and PPDK in maize, wild-type and transgenic CK rice was followed from 6:45 am (before lights were turned on) to 4 pm (Figure 1A and B, respectively). The diurnal activity of PEPC in typical C_4 plants shows an increase in activity during the first few hours of light exposure, which is expected, by the activative phosphorylation of the enzyme by phospho*enol*pyruvate carboxylase kinase (Vidal and Chollet, 1997). This pattern is seen in the maize control and in the transgenic CK rice. Wild-type rice shows very low levels of activity and very little activation. PEPC enzyme activity was significantly higher in the CK transgenic rice compared to wild-type at all time intervals ($p < 0.001$, $p < 0.001$, $p = 0.003$, $p = 0.01$, $p < 0.001$, $p < 0.001$, respectively).

PPDK is light activated by phosphorylation of a histidine residue and phosphorylation (active) and dephosphorylation (inactive) of threonine by ADP (Kanai and Edwards, 1999). The maize control shows an increase in activity over the course of the day. The CK transgenic plant, while having lower PPDK activity than maize, shows a slight increase over the course of the day while the wild-type rice shows very low levels of activity and very little activation. PPDK enzyme activity was significantly higher in the CK transgenic rice compared to wild-type at all time intervals ($p = 0.05$, $p = 0.01$, $p = 0.08$, $p = 0.01$, $p = 0.05$, $p = 0.01$, respectively).

Enzyme localization and activity

Analysis of enzyme activity was made in different organs (leaf, sheath, stem, root and floret) from maize, wild-type rice and transgenic CK rice (Figure 2). Western blots are also shown for the two enzymes in different organs in the CK rice. In maize, the

greatest PEPC enzyme activity is seen in the leaf and secondly the sheath (Figure 2A) and greatest PPDK enzyme activity is seen in the leaf and secondly the floret (Figure 2B). In wild-type rice, PEPC levels are very low in all organs with the highest activity in the stem (Figure 3A). PPDK activity in the wild-type rice was also highest in the stem (Figure 2B). Transgenic CK rice, like maize, has high activity of PEPC (and high PEPC content as shown in the western blot) in the leaf and sheath. There is a statistically significant difference in leaf PEPC enzyme activity between the CK transgenic rice and wild-type rice (p<0.001). The PPDK activity in CK leaves, on average, was higher than in wild-type, though not statistically significant in this set of experiments (p=0.11). Enzyme activity in the roots of the transgenic CK plant is not shown due to unreliable data and documented assaying difficulties with this enzyme (Chastain et al., 2006, Edwards et al., 1985). Based on the Western blot, there is little or no PPDK activity in the roots of the transgenic CK rice.

Immunolocalization

Immunolocalization using the small subunit antibody to Rubisco shows it is localized in the chloroplasts of the mesophyll cells of both the wild-type (Figure 3A) and transgenic CK rice (Figure 3C). Although it appears more labeled in the CK rice, confocal microscopy is not absolutely quantitative. Rubisco activity, while generally higher, was not statistically significant between wild-type and CK rice (Table 1).

Immunolocalization studies with the PEPC antibody shows the enzyme is localized in the cytosol of mesophyll cells in wild-type (Figure 3B) and transgenic CK rice (Figure 3D) with much heavier labeling in the transgenic plants.

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Gas exchange

Several measures of photosynthesis were made comparing wild-type and transgenic CK rice (Table I). The transgenic rice had a lower CO_2 compensation point than wild-type under 21% O_2 (50.0 vs. 60.5) though not statistically significant (Table 1 and Figure 4). The CK rice also had a much lower CO_2 compensation point at 2% O_2 than wild-type. The transgenic CK rice and wild-type rice had similar rates of assimilation at CO_2 saturation (~1400 $\mu L\ L^{-1}$) at both 2 and 21% O_2 (Table 1 and Figure 4). However, stomatal conductance was significantly higher in transgenic CK rice compared to wild-type rice at 2% O_2 (p=0.01), which is reflected in higher, though not statistically significantly different, Ci/Ca ratios (Table 1). The carboxylation efficiency between CK and wild-type plants was not statistically significantly different at 2 or 21% O_2. Light compensation points, maximum incident quantum use efficiency, and light saturated assimilation rates were determined for the wild-type and transgenic CK plants at 21% O_2 and 360 $\mu L\ L^{-1}\ CO_2$ (Table I and Figure 6). The CK plants had significantly lower light compensation points (p=0.06), but similar quantum use efficiency and maximum rates of assimilation under saturating light and atmospheric levels of CO_2, (Table I and Figure 6). There was no statistically significant difference in respiration rates between the wild-type and transgenic CK rice (-2.5 and –1.5 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$, respectively) (extrapolated from data in inset of Figure 7).

The optimum photosynthetic temperature for the wild-type rice was 35°C and the optimum photosynthetic temperature for the transgenic CK rice was 30°C (Table I and Figure 7). However, in an analysis of response of photosynthesis to temperature, the transgenic rice had higher rates of photosynthesis than the wild-type at all temperatures

up to 35°C, which were significantly different at 15, 30 and 35°C (p=0.05, p=0.04, and p=0.10, respectively), but was significantly lower at 40°C (p=0.07) (Figure 8 inset).

Rubisco activity and protein content

Rubisco activity was measured in leaf tissue of wild-type and CK transgenic rice on a per mg chlorophyll, mg protein, and area basis (Figure 8). Although, on average, CK plants had higher Rubisco activity, there were no significant differences between the wild-type and CK rice. Also, there were no significant differences between the untransformed and transformed CK rice in chlorophyll or soluble protein content.

Leaf ^{13}C content

Wild-type and transgenic CK rice has similar leaf δ^{13}C values of –26.3 and –26.6 (no significant difference).

Leaf starch content

Leaf cross-sections of wild-type (Figure 9A) and transgenic CK rice (Figure 9B) were stained using periodic acid plus Schiff's reagent to show starch content. All starch was located in the chloroplasts of the mesophyll cells. There appears to be lower starch content in the transgenic CK line compared to the wild-type.

Harvest data

Wild-type and transgenic CK rice was grown to maturity and harvested approximately 140 days after germination. There were significantly more panicles on the CK plants compared to the wild-type (p=0.05) and while there was no significant difference (p=0.15) in the grain yield (grams of grain/plant), the mean of the CK line was over 30% higher than wild-type (Table 1).

Discussion

Rice transformed with the genes encoding PEPC and PPDK from maize (CK), while not having a functional C_4 cycle, did show beneficial changes in physiology leading to increased panicle number per plant and grain yield. Transformed CK rice had higher PEPC enzyme activity than wild-type, and on average higher PPDK, though not significantly different under all assay conditions (see Figs 1 and 2 and Materials and Methods). Also, the CK line had equivalent levels of PPDK activity to the parental homozygous PPDK rice line (data not shown). The PPDK construct used in these lines of rice was the maize cDNA with the rice *Cab* promoter. It has been shown that using the intact gene of PPDK from maize has resulted in rice lines with up to 40 fold increases in enzyme activity (Fukayama et al., 2001). Unlike rice transformed with just PEPC (PC, Chapter 2), the CK line did appear to show light activation and concomitant increase in PEPC enzyme activity over the course of the day. PPDK enzyme activity, which is also light activated, also appeared to have a diurnal pattern of activity in the transgenic CK rice. Localization of PEPC in the cytoplasm of the mesophyll cells in the CK transgenic line is expected since it is a cytosolic enzyme in C_4 plants. Due to inconsistencies in immunological staining using the techniques employed in this study, we were unable to confirm the cytosolic localization of PPDK by confocal microscopy due to substantial background labeling (results not shown).

From analysis of photosynthetic parameters, there was evidence for some positive effects in the transgenic CK rice compared to wild-type. The CK lines showed lower CO_2 compensation points and lower light compensation points. In a study on temperature dependence of photosynthesis (at atmospheric CO_2 and 2000 PPFD), CK plants also had

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higher rates of photosynthesis at 15 to 35°C. Generally, C_4 plants are at a disadvantage compared to C_3 plants at lower temperatures. Conversely, the CK plants had a significantly lower photosynthetic rate compared to the wild-type rice at the highest temperature tested (40°C). This is usually when C_4 plants show greater efficiency compared to C_3, however, 40°C is approaching lethal temperatures for photosynthesis. Additionally, there was no increase in respiration rate in CK plants, although higher respiration was observed in the PC line under atmospheric conditions (Chapter 2). There was also no difference in $\delta^{13}C$ values between transgenic CK and wild-type rice grown at atmospheric conditions. This substantiates that the C_4 cycle is not working in these plants. CK plants have, on average, a higher Ci/Ca ratio than wild-type plants under 21 and 2% O_2, which is suggestive of a higher stomatal conductance. Stomatal conductance was higher in CK than wild-type under 2% O_2, although no difference was observed under 21% O_2. Similar results were obtained with PC plants (Chapter 2).

As with PC transgenic rice, it is uncertain how transformants with these C_4 genes have a significant increase in panicle number leading to increased grain yield in the CK line compared to the wild-type rice. With similar respiration rates, Rubisco activity, and chlorophyll content, there may be other factors at work other than just increased photosynthetic rates, although higher rates of photosynthesis were observed at most temperatures and under full sunlight (2000 μmol quanta m^{-2} s^{-1}). Other studies have shown that the transgenic C_4 rice may have greater ability to deal with photo-oxidation under high light, uptake of minerals from the soil and greater drought tolerance than wild-type rice (Jiao et al., 2002). Other possibilities include the refixation of photorespired CO_2 by PEPC and the shuttling of PEP into the shikimate pathway for

synthesis of aromatic amino acid and various phenolics by the conversion of pyruvate to PEP by PPDK (Hausler et al., 2002). Additionally, it has been shown that transgenic tobacco expressing only chloroplastic PPDK has increased number of seeds (Matsuoka et al., 2001). It is suggested that overexpression of PPDK in transgenic plants may increase the photosynthetic rate in organs surrounding seeds, thus increasing yield. It is possible that one, or a combination of these factors, may be beneficial to rice production under certain conditions.

Materials and Methods

Plant material used

Transgenic CK rice used for all experiments in this paper were of the third generation, developed as reported in Ku et al., 2000, using a single parent of the line designated as C4-32-5.

Plant growth conditions

All rice, *Oryza sativa* cv. Kitaake, used were grown in one-gallon pots, filled with a 4:1 mix of potting soil and clay, in growth chambers with a 12 hour photoperiod using a step-wise increase and decrease in light intensity with a maximum of 1000 µmol quanta $m^{-2} s^{-1}$ at the top of the canopy, a 26/20 °C day/ night temperature regime and 80% humidity. Plants were maintained with constant water supply and fertilized twice weekly with fertilizer (see following formulation) and once weekly with 50 mL of 10 mM KH_2PO_4, pH 7.4. All plants used for experiments were grown from seed from the same generation and collected from plants grown collectively to reduce any chance of variation. Plants were used approximately 6-7 weeks after germination except for data on reproductive development.

Rice Fertilizer formulation

Peters Hi Phos Special   15-30-15 – 2 parts

Calcium Nitrate   15.5-0-0 – 1 part

Peters All Purpose Fert. 20-20-20 – 1 part

Excel Magnitrate   10-0-0   - 1 part

Sprint 330 (Chelated Fe)   - ½ part

Peters S.T.E.M.   – ¼ part (Soluble Trace Element Mix)

Enzyme assays and Western blot analysis

All enzyme assays were performed on crude extracts obtained by grinding leaf tissue harvested from the mid-portion of four to six newly mature leaves in the light in a cold (-80 °C) mortar and pestle with sterile acid-washed sand and a grinding medium containing 50 mM HEPES (or Tris) pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM Na-pyruvate, 10% glycerol, 5% insoluble-PVP, 5 mM iodoacetic acid, 10 mM $MgCl_2$, 1% β-mercaptoethanol. The extract was then centrifuged for 10 minutes at 14,000 x g at 4°C and the supernatant used for enzyme assay. Grinding media used for diurnal enzyme activity contained 0.1% Triton X-100.

For phospho*enol*pyruvate carboxylase (PEPC) assays, activity was measured spectrophotometrically at 340 nm by following the decrease in NADH in an assay solution containing 50 mM Tris-HCl pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.25 mM NADH, 1 mM G-6-P, 4 mM PEP, 5 mM DTT, and 12 units NAD-malate dehydrogenase at 30 °C.

For pyruvate orthophosphate dikinase (PPDK) assays, activity was measured spectrophotometrically at 340 nm by following the decrease in NADH in an assay solution containing 25 mM Hepes-KOH pH 8.0, 8 mM $MgSO_4$, 10 mM DTT, 10 mM $NaHCO_3$, 2 mM Na-pyruvate, 5 mM $(NH_4)_2SO_4$, 1 mM G-6-P, 2.5 mM K_2HPO_4, 0.2 mM NADH, 0.5 units PEPC and 2 units NAD-malate dehydrogenase. The reaction was initiated by the addition of 20 μL of 50 mM ATP.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was measured using a premix of 0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$, 50 mM DTT. Just before assaying 0.2 M $NaH^{14}CO_3$ and 20 uL extract were added and incubated at 30°C

for approximately 2 minutes. The reaction was initiated by adding 15 μL of 5.0 mM

RuBP and stopped after exactly 2 minutes by adding 50 μL of 1 N HCl/4 M Formic Acid

solution. Samples were air dried, resuspended in 50 μL distilled deionized H_2O and 10

mL of scintillation cocktail and counted.

Westerns blots were performed as described in Ku et al. (1999).

Protein assays were done on supernatants using the Bio-Rad Assay kit (Hercules,

CA) using BSA as the standard.

Leaf area measurements were made using a Licor LI-3000 portable leaf area

meter (Lincoln, NE).

Chlorophyll extraction on intact leaves was done using ethanol by the method of

Knudson et al. (1977), and by ethanol from leaf extracts obtained from grinding with

mortar and pestle.

Immunolocalization

Immunolocalization was done by fixing leaf tissue samples in 1.25%

glutaraldehyde/2% paraformaldehyde in 50 mM Pipes overnight at 4 °C, dehydrating in a

series of alcohol dilutions and then embedded into LR White resin (Electron Microscopy

Sciences, Fort Washington, Pennsylvania, USA). Samples were cut at 100 nm using a

Reichert R Ultracut (Reichert-Jung GmbH, Heidelberg, Germany), placed on slides and

dried. The slides were blocked with TBST+BSA (10 mM Tris-HCl, 150 mM NaCl, 0.1%

v/v Tween 20, 1% w/v bovine serum albumin, pH 7.2) for one hour and incubated in

either maize anti-Rubisco (1:500 dilution) or anti-PEPC (1:1000 dilution) antibodies.

After a series of rinses, they were incubated in 5 nm gold conjugated goat anti-rabbit

antibody. After thorough rinsing, the slides were silver-enhanced per manufacturer's

instructions (Amersham, Arlington Heights, Illinois, USA) and stained with 0.5% (w/v) Safrinin-O for 30 seconds. Confocal images were obtained on a Biorad 1024 MRC using the 20x objective at 2x electronic zoom and 10% laser power.

Gas exchange

Gas exchange measurements were obtained from three independent plants using two fully expanded leaves from plants that had not yet entered the reproductive stage using the FastEst gas system (FastEst, Tartu, Estonia); described in detail in Laisk and Oja, 1998) with a Licor LI-6251 infra-red gas analyzer (Lincoln, NE). Input gas humidity was adjusted for each temperature based on calculation of mV to relative humidity to maintain 80% humidity. Assimilation rates, determination of Ci, light intensity (PPFD) and leaf temperature were determined per Laisk and Loreto (1996).

Leaf ^{13}C content

Plant material, collected from mature plants, was dried at 60°C for 36-72 h then ground to a fine powder. Subsamples (1.5-2.0 mg) were analyzed by continuous flow isotope ratio mass spectrometry. A Eurovector elemental analyzer was used to combust samples and separate the resulting carbon dioxide gas, which was then analyzed for ^{13}C/^{12}C using a Micromass Isoprime mass spectrometer. Results are expressed as $\delta^{13}C = 1000$ x $(R_{sample} - R_{standard})/(R_{standard})$, where $R_{standard}$ is Peedee belemnite (Craig, 1957). The precision based on replicate plant samples was generally in the range of 0.01-0.02 per mil.

Starch staining

Starch staining was done on LR White embedded leaf samples with periodic acid for one hour, rinsed and dried then stained with Schiff's reagent for one hour and dried.

Images were obtained at 100x magnification using a light-microscope. Samples were collected simultaneously at mid-day.

Statistical analyses

All statistics were obtained with SigmaStat 2.03 using one-way ANOVAs, Tukey tests and Student t-tests. Transformations were employed to correct for heterogeneity when necessary. Comparisons were considered statistically significant for p values <0.1.

Table legends:

Table I: Comparison of physiological parameters between wild-type *Oryza sativa* cv. Kitaake (WT) and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK). Results are average and standard error of three independent replicates. CO_2 compensation points were calculated from initial slopes of three separate experiments of A vs. Ci curves. Light compensation points were also calculated in this way from A vs. PPFD curves. Key: A=Assimilation rate (μmol CO_2 m^{-2} s^{-1}), Ci=intercellular CO_2, Ca=atmospheric CO_2, n.d.=no data.

Table 1

Parameter	wild-type		CK	
	21% O_2	2% O_2	21% O_2	2% O_2
CO_2 compensation point ($\mu L\ L^{-1}$)	60.5±12.2	29.4±11.0	50.0±1.6	0
Carboxylation efficiency ($mol\ m^{-2}\ s^{-1}$)	0.09±0.01	0.12±0.02	0.07±0.01	0.13±0.01
CO_2 saturated rate of A ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	53.6±2.1	61.7±4.1	54.7±1.2	65.8±3.2
Ci/Ca at max rates of A	0.56±0.16	0.65±0.16	0.70±0.03	0.72±0.06
Stomatal conductance at max rates of A (units) (p=0.01 at 2% O_2)	0.16±0.09	0.07±0.02	0.13±0.01	0.17±0.02
light compensation point ($\mu mol\ quanta\ m^{-2}\ s^{-1}$) (p=0.06)	42.3±3.8	n.d.	24.4±5.6	n.d.
Maximum incident quantum use efficiency	0.05±0.01	n.d.	0.04±0.01	n.d.
light saturated rate of A ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	25.8±4.8	n.d.	28.3±2.6	n.d.
Optimum photosynthetic temperature (°C)	35	n.d.	30	n.d.
Rubisco activity ($\mu mol\ mg\ Chl^{-1}\ min^{-1}$)	1.7±0.27	n.d.	2.3±0.35	n.d.
Rubisco activity ($\mu mol\ mg\ protein^{-1}\ min^{-1}$)	0.17±0.02	n.d.	0.23±0.04	n.d.
PEPC activity ($\mu mol\ mg\ protein^{-1}\ min^{-1}$) (p<0.001)	0.05±0.002	n.d.	2.01±0.01	n.d.
PPDK activity ($\mu mol\ mg\ protein^{-1}\ min^{-1}$)	0.01±0.003	n.d.	0.02±0.002	n.d.
Leaf $\delta\ ^{13}C$	-26.4±0.2	n.d.	-26.7±0.1	n.d.
panicle number per plant (p=0.05)	25.0±2.7	n.d.	32.7±3.8	n.d.
grain yield (g per plant)	36.7±11.2	n.d.	48.7±1.7	n.d.

Figure legends:

Figure 1: Diurnal enzyme assay of A) phospho*enol*pyruvate carboxylase (PEPC) and B) pyruvate orthophosphate dikinase (PPDK) in *Zea mays* (maize), wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize genes for PEPC and PPDK (CK). Enzyme activity was followed over the course of the day with the lights turning on at 7:00 am. Data represents the mean and standard error of three independent replicates.

Figure 2: Comparison of A) phospho*enol*pyruvate carboxylase (PEPC) and B) pyruvate orthophosphate (PPDK) enzymes by protein amount via Western blot and activity in various organs of *Zea mays* (maize), wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize genes for PEPC and PPDK (CK). Western blot is for CK rice only. L=leaf, S=sheath, ST=stem, R=root, F=floret. Data represents the mean and standard error of three independent replicates.

Figure 3: Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho*enol*pyruvate carboxylase (PEPC) immunolocalization in wild-type *Oryza sativa* cv. Kitaake (WT) A and B, respectively and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK) C and D, respectively. Confocal images were obtained using 20% laser power and 2x electronic magnification.

Figure 4: Oxygen sensitivity in wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK). Gas exchange conditions were 28°C, 80% relative humidity, 2000 μmol quanta m^{-2} s^{-1} at either 21% O_2 or 2% O_2. Data represents the mean and standard error of three independent replicates.

Figure 5: Carboxylation efficiency in wild-type *Oryza sativa* cv. Kitaake (WT) and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK). Gas exchange conditions were A) 2 and B) 21% O_2, 28°C, 80% relative humidity, and 2000 μmol quanta m^{-2} s^{-1}. Data represents the mean and standard error of three independent replicates.

Figure 6: Light response curves from wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK). Inset graph shows the initial slope of the curve used to determine quantum use efficiency. Gas exchange conditions were 28°C, 80% relative humidity, 360 μL L^{-1} CO_2 and 21% O_2. Data represents the mean and standard error of three independent replicates.

Figure 7: Photosynthetic temperature response of wild-type *Oryza sativa* cv. Kitaake (WT), and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK). Inset graph shows the percent increase in the assimilation rate of PC over wild-type at each temperature. Gas exchange

conditions were 360 μL L^{-1} CO_2, 80% relative humidity, 2000 μmol quanta m^{-2} s^{-1} and 21% O_2. Data represents the mean and standard error of three independent replicates.

Figure 8: A comparison of Rubisco activity on a chlorophyll, protein and leaf area basis and chlorophyll and soluble protein content in wild-type *Oryza sativa* cv. Kitaake (WT) and transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK). The average and standard error of three independent replicates are shown. Values for μmol CO_2 fixed m^{-2} s^{-1} were divided by 10 and mg Chl cm^{-2} multiplied by 10 to fit scale.

Figure 9: Comparison of starch staining in A) wild-type *Oryza sativa* cv. Kitaake (WT), and B) transgenic rice over-expressing the maize genes for phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase (CK).

Figure 1.

A
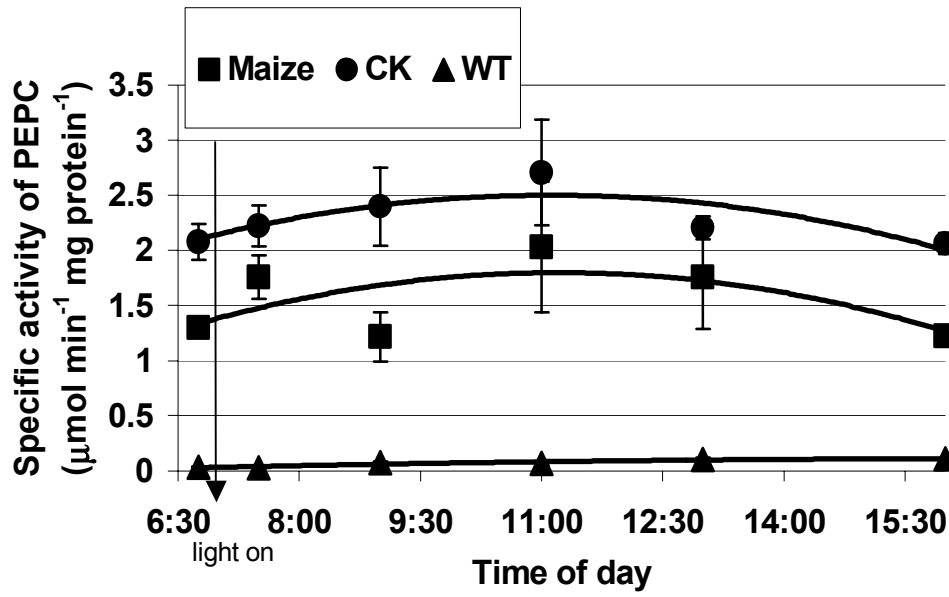
B
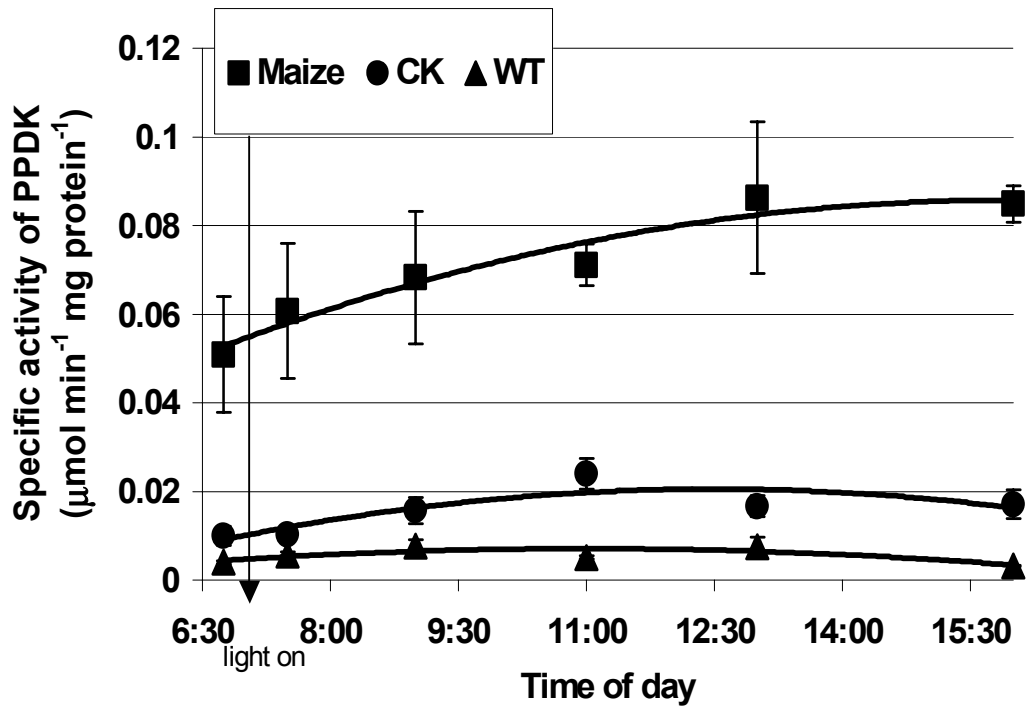
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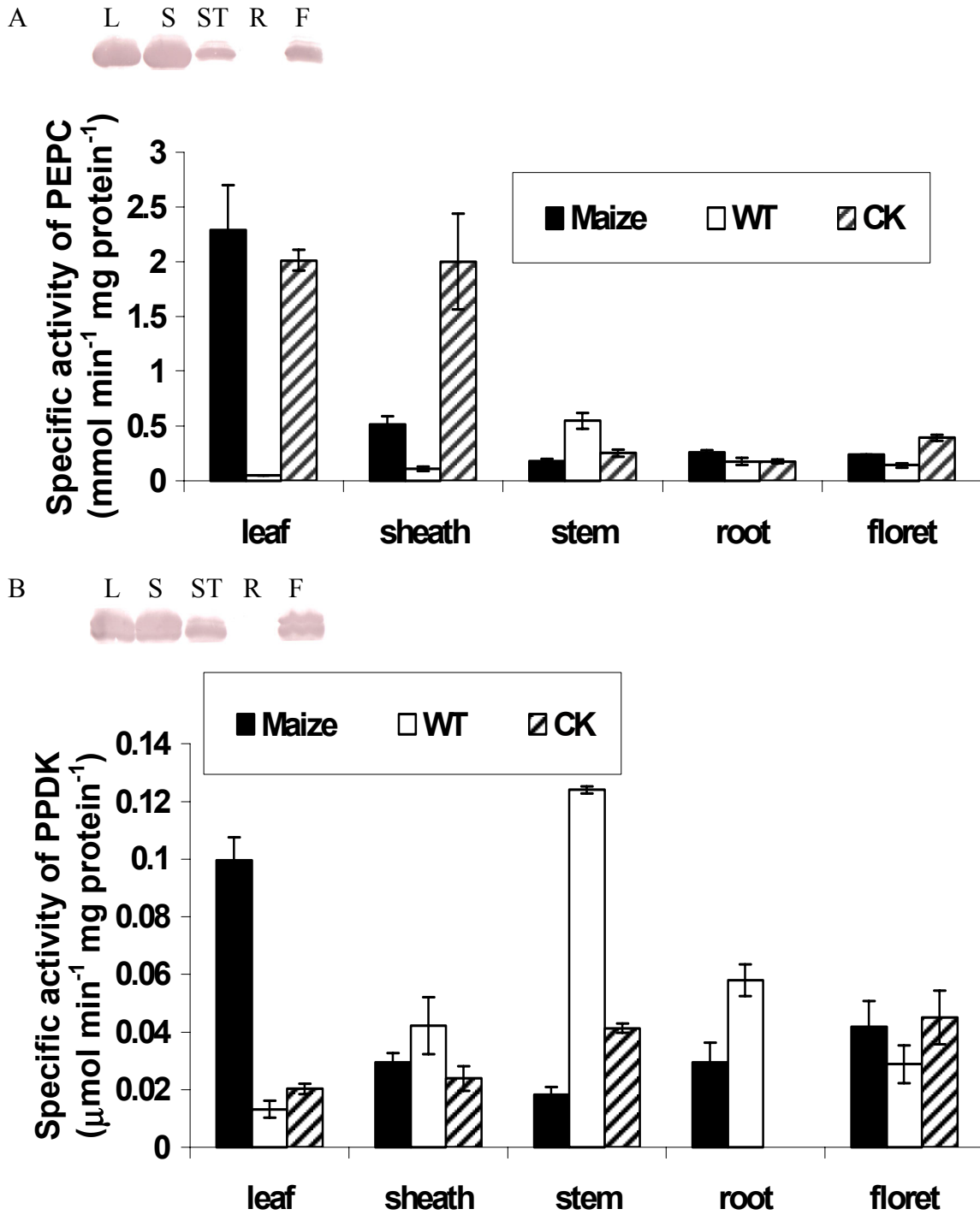
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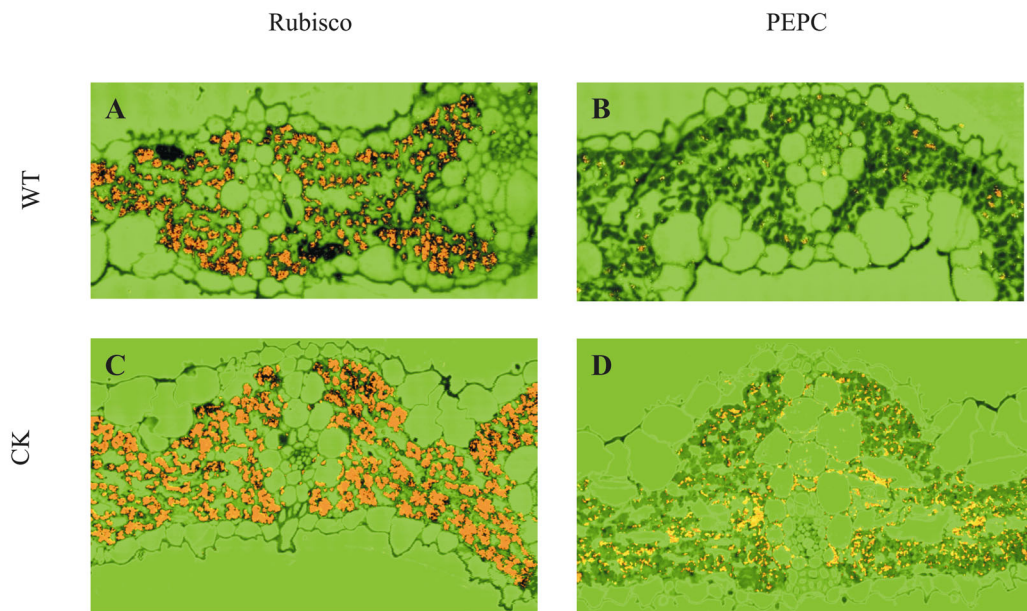
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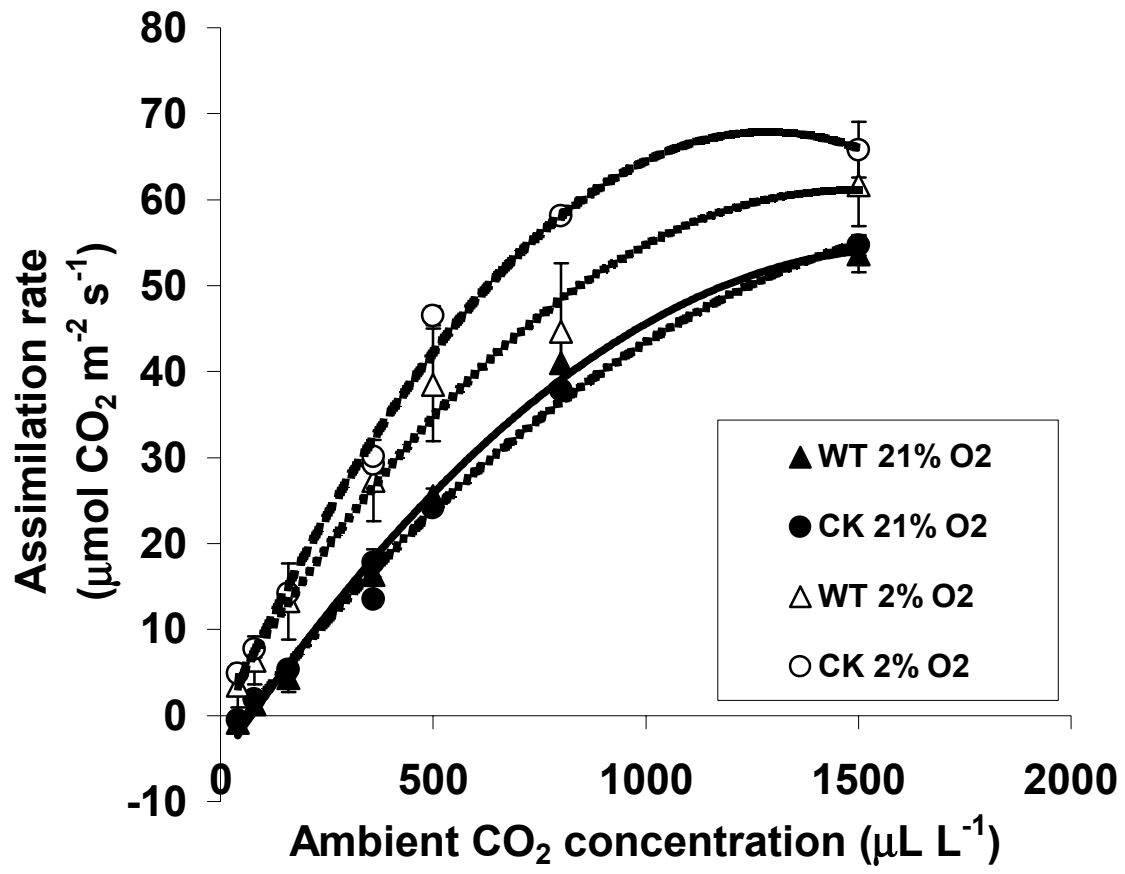
Figure 5.

A
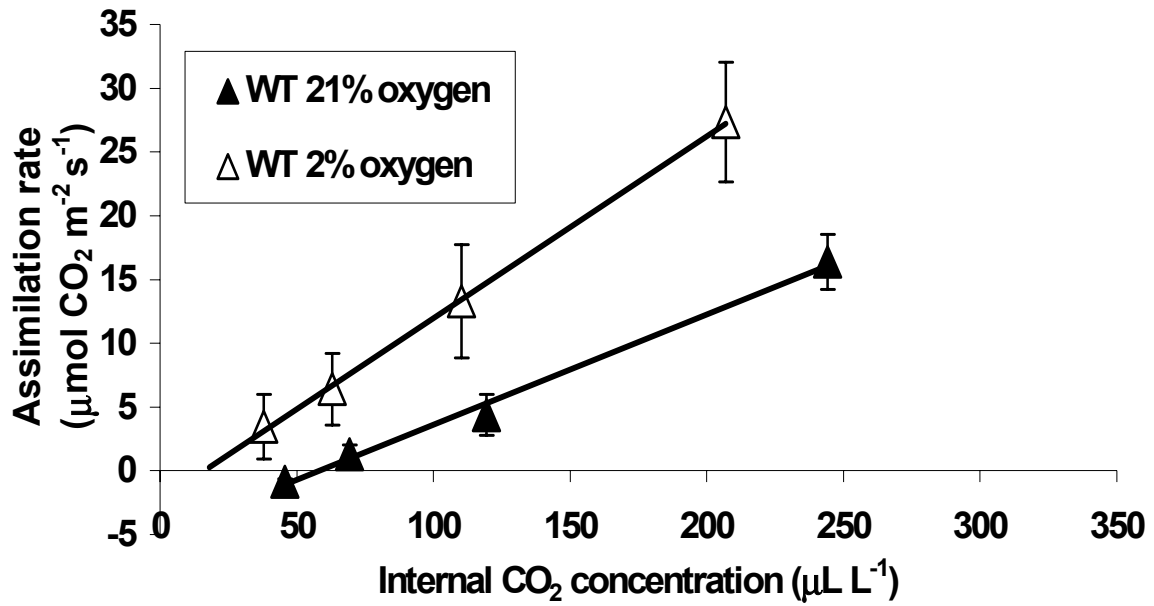
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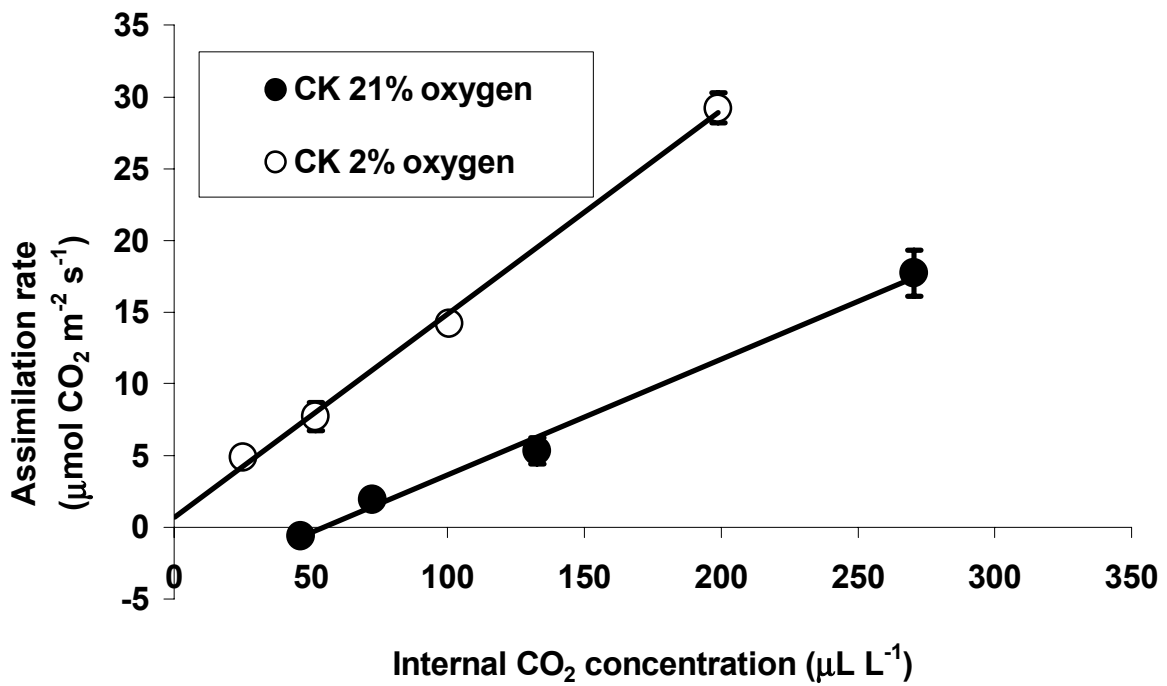
Figure 6.

Figure 7.

Figure 8.

Legend:
- ■ $\mu mol\ CO_2\ mg\ Chl^{-1}\ min^{-1}$
- ■ $\mu mol\ CO_2\ mg\ protein^{-1}\ min^{-1}$
- ■ $(\mu mol\ CO_2\ m^{-2}\ s^{-1})/10$
- □ $mg\ protein\ cm^{-2}$
- ▨ $mg\ Chl\ cm^{-2}*10$
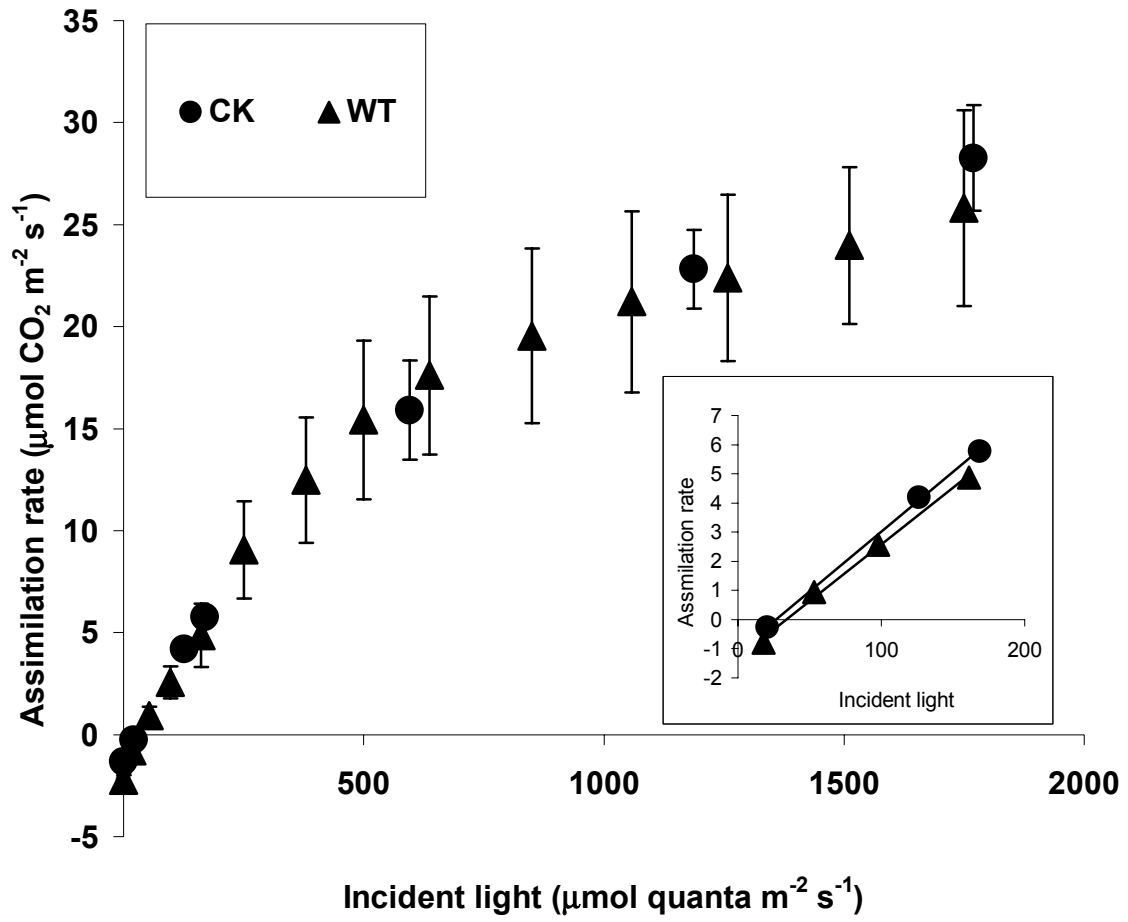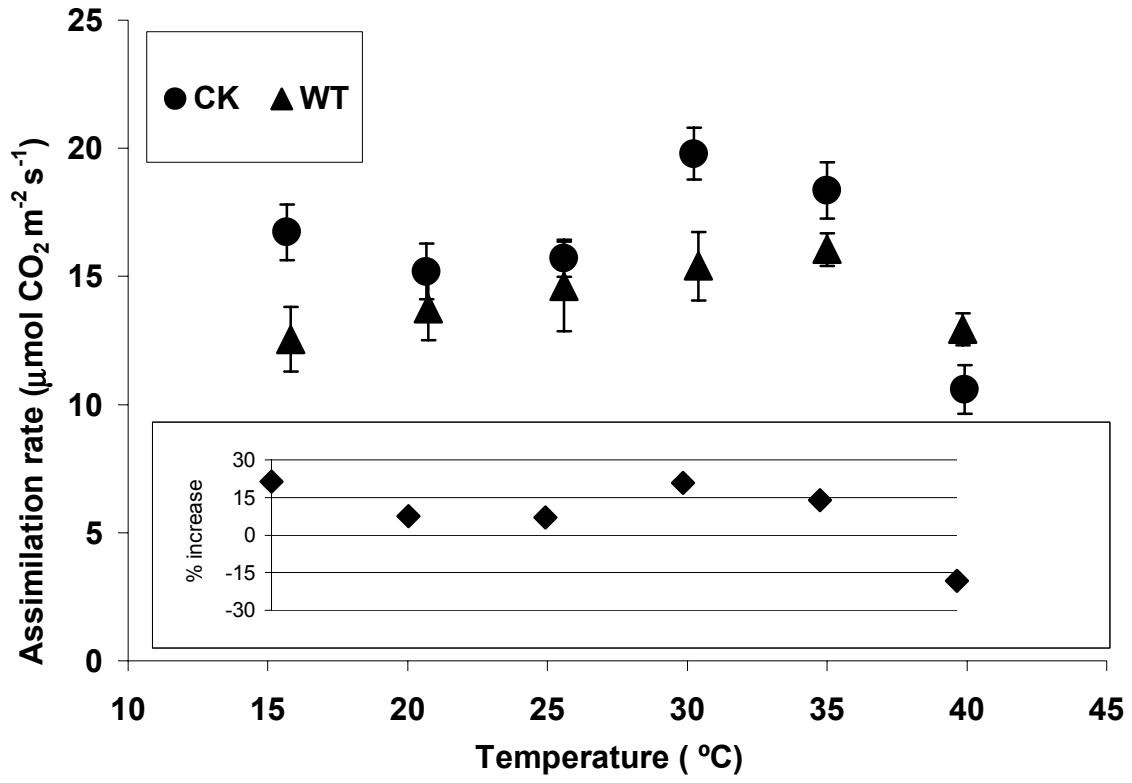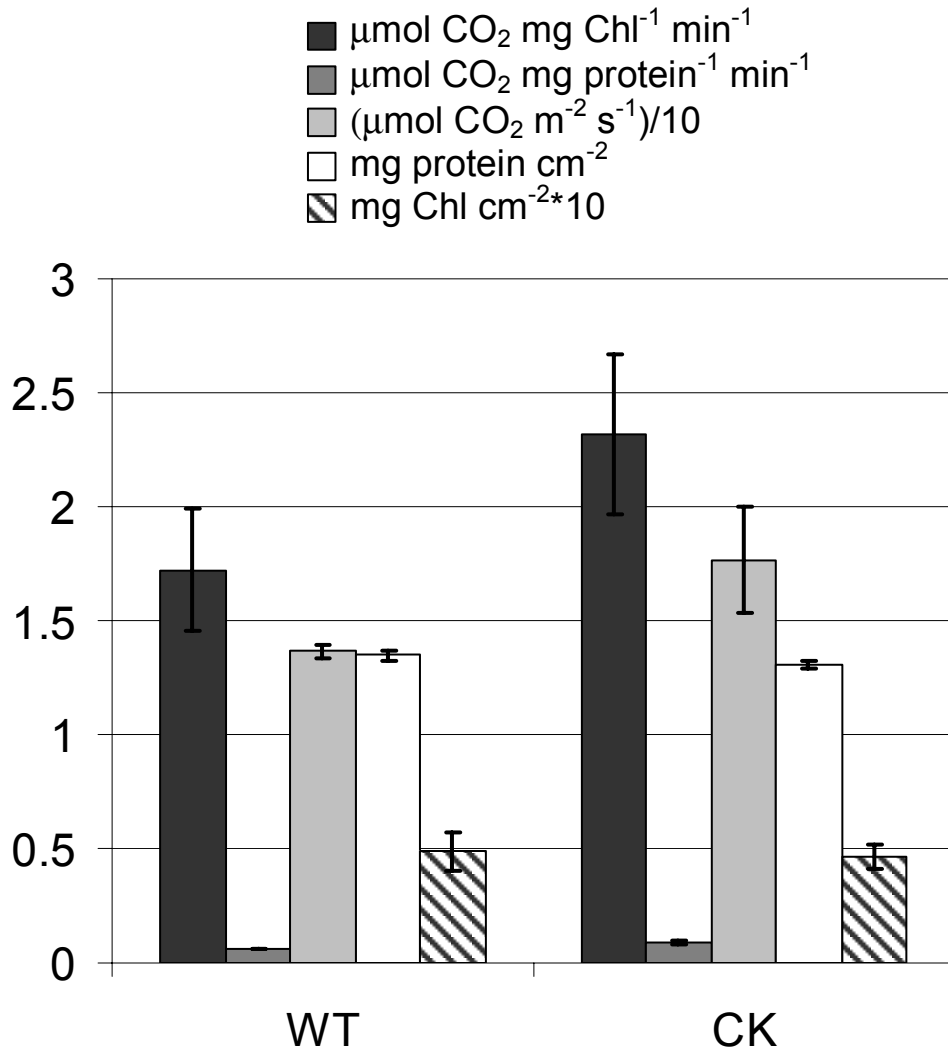
Figure 9.

A

B
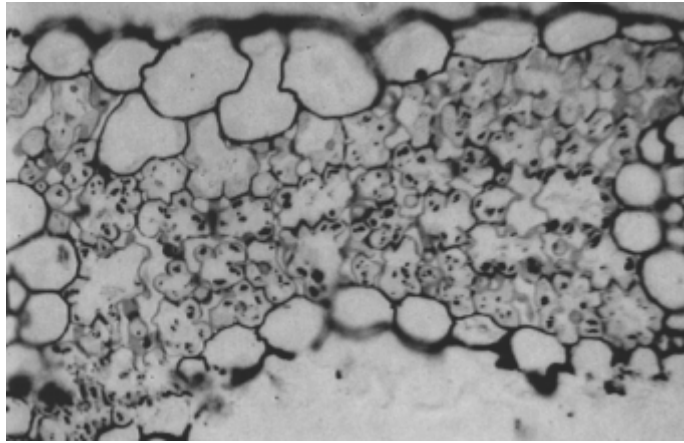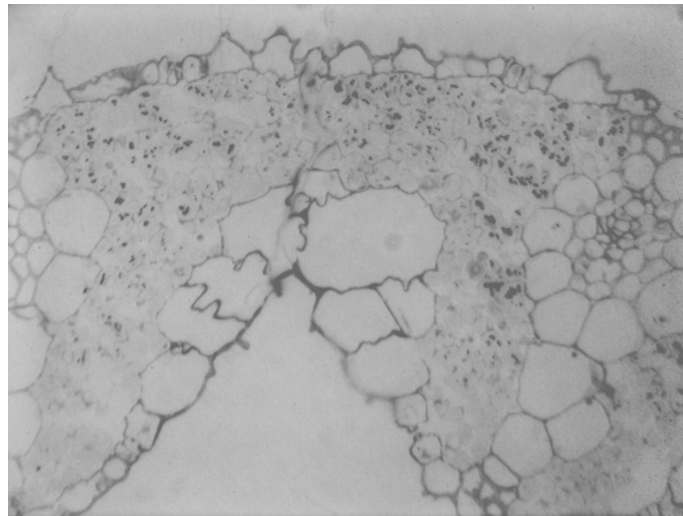
LITERATURE CITED

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. Aquat Bot 34: 233-286

Chastain CJ, Heck JW, Colquhoun TA, Voge DG, Gu XY (2006) Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (*Oryza sativa*) seeds. Planta 224 (4): 924-934

Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. Geochim Cosmochim Acta 12: 133-149

Edwards GE, Nakamoto H, Burnell JN, Hatch MD (1985) Pyruvate, Pi dikinase and NADP-malate dehydrogenase in C_4 photosynthesis: Properties and mechanism of light/dark regulation. Ann Rev Plant Physiol 36: 255-286

Edwards GE, Franceschi VR, Voznesenskaya EV (2004) Single cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Bio 55, 173-196

Fukayama H, Tsuchida H, Agarie S, Nomura M, Onodera H, Ono K, Lee BH, Hirose S, Toki S, Ku MSB, Makino A, Matsuoka M, Miyao M (2001) Significant accumulation of C_4–specific pyruvate, orthophosphate dikinase in a C_3 plant, rice. Plant Physiol 127: 1136-1146

Hausler RE, Hirsch HJ, Kreuzaler F, Peterhansel C (2002) Overexpression of C_4-cycle enzymes in transgenic C_3 plants: a biotechnical approach to improve C_3 photosynthesis. J Exp Bot 53(369): 591-607

Jiao D, Huang X, Li X, Chi W, Kuang T, Zhang Q, Ku MSB, Cho D (2002) Photosynthetic characteristics and tolerance to photo-oxidation of transgenic rice expressing C_4 photosynthetic enzymes. Photosynth Res 72: 85-93

Kanai R, Edwards GE (1999) The biochemistry of C_4 photosynthesis. In RF Sage, RK

Monson, eds, *C_4 plant biology*, Academic Press, San Diego, pp.49-88

Knudson LL, Tibbitts TW, Edwards GE (1977) Measurement of ozone injury by

determination of leaf chlorophyll concentration. Plant Physiol 60: 606-608

Ku MSB, Agarie S, Nomura M, Fukayama H, Tsuchida H, Ono K, Hirose S, Toki S,

Miyao M, Matsuoka M (1999) High-level expression of maize

phospho*enol*pyruvate carboxylase in transgenic rice plants. Nat Biotechnol 17:

76-80

Ku MSB, Cho D, Ranade U, Hsu TP, Li X, Jiao DM, Ehleringer J, Miyao M, Matsuoka

M (2000) Photosynthetic performance of transgenic rice plants overexpressing

maize C_4 photosynthesis enzymes. In JE Sheehy, PL Mitchell, B Hardy, eds,

Redesigning rice photosynthesis to increase yield. Proceedings of the workshop

on the quest to reduce hunger: redesigning rice photosynthesis. Elsevier Science

B.V., IRRI and Amsterdam, pp 193-204

Laisk A, Loreto F (1996) Determining photosynthetic parameters from culm CO_2

exchange and chlorophyll fluorescence. Plant Physiol 110: 903-912

Laisk A, Oja V (1998) Dynamics of culm photosynthesis. Commonwealth Scientific and

Industrial Organization Publishing, Collingwood, Australia

Leegood RC (2002) C_4 photosynthesis: principles of CO_2 concentration and prospects for

its introduction into C_3 plants. J Exp Bot 53 (369): 581-590

Matsuoka M, Furbank RT, Fukayama H Miyao M (2001) Molecular engineering of C_4

photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 52: 297-314

Raines CA (2006) Transgenic approaches to manipulate the environmental responses of

the C_3 carbon fixation cycle. Plant Cell Environ 29: 331-339

Sage RF, Pearcy RW (1987) The nitrogen use efficiency of C_3 and C_4 plants. I. Leaf

nitrogen, growth and biomass partitioning in *Chenopodium album* (L.) and

Amaranthus retroflexus (L.). Plant Physiol 84: 954-958

Vidal J, Chollet R (1997) Regulatory phosphorylation of C_4 PEP carboxylase. Trends

Plant Sci 2(6): 230-237

von Caemmerer S (2003) C_4 photosynthesis in a single C_3 cell is theoretically inefficient

but may ameliorate internal CO_2 diffusion limitations in C_3 leaves. Plant Cell

Environ 26: 1191-1197

CHAPTER FOUR

The diversity and plasticity of C$_4$ photosynthesis in *Eleocharis* (Cyperaceae)

Abstract

The genus *Eleocharis* contains many amphibious species and displays diversity with regards to photosynthetic mechanism (C_3, C_4 or C_3-C_4 intermediates). A unique feature of *Eleocharis* is the plasticity of some species in their photosynthetic mechanism in response to the environment. *E. baldwinii* is C_4-like in terrestrial habitat, exhibiting O_2 inhibition of photosynthesis with Rubisco expressed in both mesophyll and bundle sheath cells and PEPC strictly in the mesophyll cells, but it switches to C_3-C_4 intermediacy when submerged. In addition to *Eleocharis vivipara* type 1 (which switches from C_4-like to C_3), as reported by Ueno and colleagues, two other photosynthetic types examined in this study were shown to have different responses to growth in either terrestrial or submerged conditions. *E. vivipara* type 2 is a typical C_4 plant in the terrestrial habitat, but becomes a C_3-C_4 intermediate under submerged conditions. Most interesting is *E. vivipara* type 3. Terrestrially, it is a C_3-like intermediate, but when submerged the $\delta^{13}C$ value increases to -6.7‰, indicating its use of bicarbonate as a major carbon source. The submerged form of this plant exhibited about 3 times higher photosynthetic O_2 evolution rate, compared to the C_3 species. These *Eleocharis* species possess different molecular switches for regulating C_4 gene expression in response to environmental stimuli both between different species, and in *E. vivipara* among different populations. The apparent acquisition of a bicarbonate transport system by *E. vivipara* type 3 while submerged represents a novel adaptation to low CO_2 availability.

Introduction

   Eleocharis (Cyperaceae) includes approximately 252 species with a worldwide

distribution (Gonzalez-Elizondo and Peterson, 1997; Simpson et al. in prep, World

Checklist of Cyperaceae). The majority of species in *Eleocharis* are restricted to wet or

submerged zones of permanent or ephemeral wetland and riparian habitats. Previous

studies have suggested that *Eleocharis* species have diverse mechanisms of fixing carbon,

including C_3, C_4, and C_3-C_4 intermediate photosynthetic types (Agarie et al., 2002; Bruhl

et al., 1987; Bruhl, 1995; Bruhl and Perry, 1995; Ueno, 2004) and that the diversification

of C_3-C_4 intermediate and C_4 physiological processes from a C_3 ancestor have occurred at

least twice within *Eleocharis* (Roalson and Friar, 2000; Roalson, unpubl. data).

   Eleocharis is of particular interest for studying the evolution of photosynthetic

pathways and morphological forms for several reasons. In terms of photosynthetic

pathway evolution, the Cyperaceae represents the second largest diversification. C_4

species have been identified in nineteen angiosperm families (Sage, 2004). Of all

recognized C_4 plants, 61% of them belong to the Poaceae while the Cyperaceae

represents 18% (Sage et al., 1999). Given current hypotheses of phylogenetic

relationships within Cyperaceae (Muasya et al., 1998), there appears to have been a

minimum of four origins of the C_4 pathway in the 28 genera of Cyperaceae that include

C_4 species. While a significant effort to understand the evolution of the C_4 pathway has

been made in grasses (Poaceae), chenopods (Chenopodiaceae), and *Flaveria*

(Asteraceae), very little effort has been made in family Cyperaceae, with most studies in

the family focusing on *E. vivipara* Link (Agarie et al., 1997; Agarie et al., 2002; Bruhl,

1995; Bruhl and Perry, 1995; Takeda et al., 1985). In addition to representing a

significant portion of all C_4 plants, the Cyperaceae also include two of the three primary C_4 biochemical sub-types, NADP-ME and NAD-ME (Bruhl and Perry, 1995).

Although several other genera, such as *Panicum* (Edwards and Ku, 1987) and *Flaveria* (Edwards and Ku, 1987; Ku et al., 1991), are known to exhibit diversity in the mechanism of photosynthesis, the unique feature of *Eleocharis* is that several species have been shown to exhibit varying degrees of C_4 characteristics and genetic plasticity in changing photosynthetic mechanism and culm anatomy depending on growth habitat. No other C_4 species are known to possess this phenotypic plasticity. Therefore, *Eleocharis* provides an excellent opportunity for studying photosynthetic adaptation to the environment and the underlying molecular mechanisms that switch on or off the expression of biochemical and structural C_4 genes under different growing conditions. Previous studies by Ueno and colleagues demonstrated that *E. vivipara* (designated type 1 in this study) expressed C_4-like characteristics in the terrestrial form and C_3 in the submerged form without Kranz anatomy (Agarie et al., 1997; Agarie et al., 2002; Uchino et al., 1998; Ueno, 1996a, 1996b; Ueno, 1998; Ueno, 2001; Ueno, 2004; Ueno et al., 1988, 1989) whereas *E. baldwinii* (Torrey) Chapman expressed C_4 characteristics in the terrestrial form but C_3-C_4 intermediate in the submerged form (Uchino et al., 1995; Ueno, 2004). In contrast, *E. retroflexa* (Poir.) Urb. ssp. *chaetaria* (Roem. & Schult.) Koyama, which has complete C_4 characteristics in the terrestrial form, expresses C_4 biochemical traits with Kranz-like anatomy underwater (Ueno and Samejima, 1989; Ueno and Wakayama, 2004). This demonstrates a great diversity in photosynthesis in the genus and most uniquely the genetic plasticity in modifying the photosynthetic mode in adaptation to growth environment. Studies on other members of the family Cyperaceae

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found that, while there is variability in the Kranz anatomy in C_4 members, they are all of the NADP-ME decarboxylating sub-type (Ueno et al., 1986). Subsequent studies discovered that the C_4 *Eleocharis* species use NAD-malic enzyme as the major C_4 acid decarboxylation enzyme (Bruhl et al., 1987; Ueno and Samejima, 1989; Ueno, 2004).

Several studies have focused on the anatomical characterization within the C_4 Cyperaceae (Bruhl et al., 1987; Soros and Dengler, 2001; Uchino et al., 1998; Ueno, 1996b; Ueno, 1998). The variations include rhynchosporoid, chlorocyperoid, fimbristyloid and eleocharoid. Within the genus *Eleocharis* only fimbristyloid and eleocharoid have been described. Typical C_4 Kranz anatomy is characterized by vascular tissue being completely encircled by bundle sheath cells (BSC), which in turn are surrounded by mesophyll cells (MC). The C_4 acid decarboxylating enzymes and Rubisco are strictly compartmentalized into the BSC, while the primary carboxylating enzyme PEPC is in the cytosol of the MC. In Cyperaceae there is an exception to this arrangement by the presence of a mestome sheath between the MC and BSC (Soros and Dengler, 2001). These cells are nonphotosynthetic, generally suberized, and they may have an impact on diffusion and signaling (Soros and Dengler, 2001). Fimbristyloid Kranz anatomy, described in *E. vivipara* type 1 (Bruhl et al., 1987; Soros and Dengler, 2001; Ueno, 1998), is characterized by BSC being interrupted by metaxylem vessels. Eleocharoid anatomy is characterized by an unbroken "wreath" of BSC surrounding the vasculature and is found in all other described C_4 *Eleocharis* species.

While detailed photosynthetic pathway characterizations have been made of a small number of *Eleocharis* species, very little is known about the photosynthetic pathway in the majority of species. Most species that have been examined for

photosynthetic mechanism have been characterized using the presence of Kranz anatomy and carbon isotope ratios from herbarium collections (Ueno et al., 1989). Given the plasticity of photosynthetic types within *Eleocharis* under different growing conditions and the often unclear descriptions of growing conditions of herbarium specimens, detailed studies of more species are necessary to characterize the diversity and phenotypic plasticity of photosynthetic mechanisms in the genus.

In this study, we conducted photosynthetic, biochemical and anatomical analyses of culms of several *Eleocharis* species grown in submerged and terrestrial conditions, and compared them with other well-characterized C_4 species. Our data show that three *Eleocharis* photosynthetic types, exhibiting varying degrees of C_4 photosynthesis in terrestrial form, either switch to C_3-C_4 or C_3 photosynthesis with Kranz-like or non-Kranz anatomy, respectively, when grown submerged. Most interesting, *Eleocharis vivipara* type 3 apparently uses bicarbonate rather than CO_2 as a major carbon source when submerged.

Results

Culm anatomy

Culms of terrestrial and submerged *Eleocharis* differ significantly in anatomy between species (*E. baldwinii* and *E. vivipara*) and between individuals (*E. vivipara* type 2 and type 3) depending on growth conditions (Figure 1). *Eleocharis erythropoda* is an exception as it has large, empty bundle sheath cells (BSC) and two layers of mesophyll cells (MC) containing chloroplasts when grown either terrestrially (A) or submerged (E). The terrestrial form of *E. vivipara* type 3 exhibits Kranz anatomy with densely-packed chloroplasts in both MC and BSC (B), but when grown submerged the previously dense BSC now appear empty and the MC chloroplasts appear peripherally arranged – a non-Kranz anatomy (F). Terrestrial *E. baldwinii* also possesses Kranz anatomy and has numerous chloroplasts in its BSC (C), which appear peripherally arranged when grown submerged (G). Both *E. vivipara* type 3 and *E. baldwinii* have open arrangements of their BSC, described as fimbristyloid (Bruhl et al., 1987; Soros and Dengler, 2001; Ueno, 1996b), which is characterized in the Cyperaceae as having metaxylem vessels interrupting the BSC's wreath-like arrangement. In contrast, the culms of terrestrial *E. vivipara* type 2 exhibits well-developed Kranz anatomy with a closed arrangement of BSC relative to the vasculature and these BSC contain numerous chloroplasts (D). This arrangement is described as eleocharoid (Bruhl et al., 1987; Soros and Dengler, 2001). When grown submerged, there are fewer chloroplasts in the BSC, which are peripherally arranged, as are the chloroplasts in the MC (H). Species with Kranz-like or Kranz anatomy exhibit significant alterations in structure when submerged, i.e., changing to non-Kranz or less developed Kranz-like structure. It is also interesting to note the

differentiation in Kranz anatomy among the three types of *E. vivipara*, with types 1 and 3 showing fimbristyloid and type 2 showing eleocharoid structure.

Enzyme activity and culm $\delta^{13}C$

Key enzymes involved in C_3 (Ribulose 1,5 bisphosphate carboxylase/oxygenase [Rubisco]) and C_4 photosynthesis (phospho*enol*pyruvate carboxylase [PEPC], pyruvate orthophosphate dikinase [PPDK], NADP-malic enzyme [NADP-ME], NAD-malic enzyme [NAD-ME] and phospho*enol*pyruvate carboxykinase [PCK]) were assayed in the photosynthetic culms of terrestrial and submerged plants (Table 1). The terrestrial form of *E. erythropoda* had the lowest activity for all C_4 photosynthetic enzymes and the highest activity for Rubisco. When submerged, the activities of PEPC, PPDK and Rubisco decreased (Rubisco reduction statistically significant, $p<0.001$). Both terrestrial and submerged forms of *E. erythropoda* have very similar culm $\delta^{13}C$ values, -30.8 and –30.5‰, respectively, within the range for a typical C_3 plant. On the other hand, the terrestrial form of *E. vivipara* type 2 had the highest activities of most C_4 enzymes (e.g., PEPC, PPDK, NAD-ME) among the four *Eleocharis* photosynthetic types examined, lower Rubisco activity relative to *E. erythropoda*, and a $\delta^{13}C$ value of –13.2‰. When *E. vivipara* type 2 was submerged, however, the activity of PEPC, PPDK and Rubisco decreased significantly ($p<0.001$), and NAD-ME increased significantly ($p=0.02$), and the submerged form had an intermediate culm $\delta^{13}C$ value (-18.9‰). *Eleocharis baldwinii* has high PEPC and NAD-ME activities and a culm $\delta^{13}C$ content of –15.0‰ when grown terrestrially. Rubisco activity is lower than that of *E. erythropoda* but significantly higher than that of *E. vivipara* type 2 ($p<0.001$). When submerged, the activity of all of these enzymes decreased significantly ($p<0.001$ for all) with the exception of NADP-ME,

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which increased ($p<0.001$), and the submerged form possessed a culm $\delta^{13}C$ value of –

16.6‰. The terrestrial *E. vivipara* type 3 had even lower C_4 enzyme activities and

intermediate $\delta^{13}C$ value (-16.7‰). When submerged, the activity of the C_4 enzymes

decreased greatly (with the exception of NADP-ME which increased), with the activity of

PPDK decreasing to a level comparable to that of *E. erythropoda* (PPDK, NAD-ME and

Rubisco reductions significant at $p<0.001$, $p=0.01$, and $p<0.001$, respectively). Unlike

the other species, the submerged form of *E. vivipara* type 3 exhibited an enriched $\delta^{13}C$

content in the culms (-6.7‰).

Western immunoblot analysis

Western immunoblot analysis for PEPC and Rubisco large subunit (LSU) was

consistent with results of enzyme activity (Figure 2 and Table 1). The terrestrial form of

E. vivipara type 2 has the highest amount of PEPC while *E. erythropoda* has the least

amount of the enzyme. *E. baldwinii* contains substantial amounts of PEPC, while *E.

vivipara* type 3 has lower levels. *E. erythropoda* has the highest amount of Rubisco, *E.

vivipara* type 2 has the least, with *E. vivipara* type 2 *E. baldwinii* having intermediate

amounts. For all species examined, expression of both PEPC and Rubisco in the

submerged forms is reduced relative to terrestrial forms, consistent with the changes in

activity of these two enzymes.

Photosynthetic rate, carboxylation efficiency, photosynthetic CO_2 compensation

point, and oxygen inhibition of photosynthesis of terrestrial forms of *Eleocharis*

Under terrestrial conditions, *E. erythropoda* had the lowest photosynthetic rate

under ambient conditions (30°C, 360 $\mu L\ L^{-1}$ CO_2, 1000 μmol quanta $m^{-2}\ s^{-1}$, 80% relative

humidity and 21% O_2) and a low carboxylation efficiency (CE; determined from the

initial photosynthetic CO_2 response curve), and *E. vivipara* type 2 the highest

photosynthetic rate and a high CE (Figure 3A and B). *Eleocharis vivipara* type 3 and *E. baldwinii* had approximately equal rates of CO_2 assimilation at 21% O_2. However, *E. baldwinii* has the highest CE among the four photosynthetic types tested. When

measured at 2% O_2, photosynthetic rates increased for all four *Eleocharis* photosynthetic

types. *E. erythropoda* showed the highest O_2 inhibition of photosynthesis (37.2%), which

was statistically significant compared to *E. baldwinii* and *E. vivipara* type 2 (p=0.004 and

p<0.001, respectively) (Figure 4A). It also has the highest photosynthetic CO_2

compensation point (Γ), which was statistically significantly compared to *E. vivipara*

type 2 (p=0.05), which had very little O_2 inhibition (3.1%) and a Γ typical of a C_4 plant.

Relative to *E. vivipara* type 2, *E. vivipara* type 3 had much higher O_2 inhibition (32.3%),

again, statistically significant compared to *E. baldwinii* and *E. vivipara* type 2 (p=0.01

and p=0.001, respectively) and an intermediate Γ, whereas *E. baldwinii* had much

reduced O_2 inhibition (12.8%) and a relatively low Γ. CO_2 compensation points,

especially in *E. erythropoda* and *E. vivipara* type 3, show large variability between

replications. The calculation of Γ is based on measurement of photosynthesis at low

levels of C_i and extrapolation to the zero intercept on the Y-axis. Between replications

within a species there is considerable variation in C_i values at a given C_a value.

Calculating C_i requires determining diffusive resistance from measurements of

transpiration rates. This suggests there is leaf-to-leaf variation, or possible errors in the

accuracy of calculating C_i under the low rates of gas exchange.

Photosynthetic rates of submerged plants

At 28^oC, 21% O_2 and 0.5 mM $NaHCO_3$ at pH 8.0 (or 11 µM CO_2 at equilibrium),

the submerged form of *E. erythropoda* had a photosynthetic O_2 evolution rate of about 40

µmol mg Chl^{-1} h^{-1}, which increased progressively to 48µmol mg Chl^{-1} h^{-1} with increasing

bicarbonate concentration up to 2.0 mM (20% increase) (Figure 5); these rates were

significantly higher than *E. baldwinii* ($p<0.001$). These rates are comparable to those

obtained with the terrestrial form (Figure 3A). The submerged forms of *E. baldwinii* and

E. vivipara type 2 had much lower rates than those obtained with their terrestrial

counterparts, but in general they all increased with increasing bicarbonate concentration.

In sharp contrast, the submerged form of *E. vivipara* type 3 had a 2-3 fold higher

photosynthetic rate compared to all of the others ($p<0.001$). On a chlorophyll basis, the

rates of the submerged form are also 2 fold higher than its terrestrial form (Figure 3A).

Intercellular localization of Rubisco and PEPC

Intercellular localization of PEPC and Rubisco was examined in the culms of

terrestrial and submerged forms of all four *Eleocharis* photosynthetic types by

immunolabeling (Figure 6). For terrestrial forms, *E. erythropoda* had high labeling of

Rubisco and low labeling of PEPC in the mesophyll cells. In contrast, *E. vivipara* type 2

labeling of Rubisco was restricted to the bundle sheath chloroplasts while labeling of

PEPC was restricted to the mesophyll cells indicating a strict intracellular

compartmentation of key C_3 and C_4 enzymes in the species. Interestingly, *E. vivipara*

type 3 and *E. baldwinii*, both having Kranz-like anatomy, lacked a strict

compartmentation of Rubisco; labeling of Rubisco was found in both mesophyll and

bundle sheath cells. However, labeling of PEPC was restricted to mesophyll cells. When

submerged, the development of Kranz anatomy and labeling of both enzymes in these species were weakened, but without changing the intracellular pattern of labeling (data not shown).

<u>Discussion</u>

In this study, we have examined the photosynthetic mechanisms in *E. erythropoda, E. baldwinii* and two populations of *Eleocharis vivipara* (*E. vivipara* type 2 and 3) using anatomical, immunolocalization, physiological and biochemical tools. These three *Eleocharis* species exhibit a gradient in types of photosynthesis when grown terrestrially, ranging from C_3 values in *E. erythropoda*, C_3-like in *E. vivipara* type 3, C_4-like in *E. baldwinii*, and typical C_4 in *E. vivipara* type 2. All the C_4 *Eleocharis* species examined so far belong to NAD-ME subtype (Bruhl et al., 1987; Ueno and Samejima, 1989; Ueno, 2004) and this turns out to be the case with the additional C_4 and C_4-like *Eleocharis* species identified in this study. The three representative C_4 species possess high PEPC activities and the relative activities of the three C_4 acid decarboxylation enzymes in each of these species support their classification into the NAD-ME C_4 subtype (Table I). Previous characterizations of *E. baldwinii* suggested it was C_4 from carbon isotope discrimination (Uchino et al., 1995) and C_4-like based on enzymatic studies (Ueno, 2004). Although the terrestrial form of *E. baldwinii* has good Kranz culm anatomy and high C_4 enzyme activities, our studies show that it lacks a strict intercellular compartmentation of Rubisco, has substantial O_2 inhibition of photosynthesis, a Γ higher than that of a typical C_4 plant, and a somewhat less enriched ^{13}C content than most typical C_4 plants. Presumably, direct fixation of atmospheric CO_2 by Rubisco in the mesophyll cells results in substantial O_2 inhibition of photosynthesis in this plant. Therefore, *E. baldwinii* might be best characterized as a C_4-like species. In comparison to *E. baldwinii*, *E. vivipara* type 3 has relatively lower activities of all key C_4 enzymes and again a lack of cellular compartmentation of Rubisco. Concurrently, it exhibits a much higher O_2

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sensitivity by its photosynthetic machinery, relative to the C_4 and C_4-like species, thus it is characterized as C_3-like. In contrast, the terrestrial *E. vivipara* type 2 has values indicative of a C_4 species as it has typical Kranz anatomy, little O_2 inhibition of photosynthesis, low Γ, a strict cellular compartmentation of Rubisco and PEPC, and a much enriched ^{13}C content. In comparison, terrestrial *E. vivipara* type 1 is C_4-like, with fimbristyloid Kranz anatomy, Rubisco is present in both MC and BSC, it has high PEPC and NAD-ME enzyme activities and $\delta^{13}C$ value between that of *E. vivipara* type 3 and *E. baldwinii* (Agarie et al., 2002; Ueno et al, 1989). As a comparison, among terrestrial plants, C_4 species have ^{13}C values of ~ -10 to –14‰ and C_3 from ~ -25 to –30‰ (Cerling, 1999). Similar differences occur in aquatic environments although the range may be greater and more extreme, while bicarbonate users have ^{13}C values that show lack of discrimination between ^{13}C and ^{12}C (Bowes and Salvucci, 1989).

Thus, for the first time three different photosynthetic mechanisms are recorded in different populations of the same species. Previously, *E. vivipara* type 1 was characterized by Ueno and colleagues from plants found in a creek near Tampa, Florida. *Eleocharis vivipara* type 2 and 3, were found further north in northern Florida and southern Georgia, respectively. Both of these populations were found growing at least partially submerged in wetlands along highways. Both *E. vivipara* type 2 and type 3 have nearly identical DNA sequences to type 1 (but distinguishable sequences from *E. baldwinii*; Roalson, unpublished) and yet they exhibit different photosynthetic mechanisms and adaptation strategies to submerged condition. Clearly, they represent different stages of C_4 evolution, with type 1 being C_4-like, type 2 a typical C_4 and type 3 a C_3-like C_3-C_4 intermediate. Current phylogenies show that these collections are the same

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species (Roalson, unpubl. data). Thus, this species may be under-going diversification of its photosynthetic machinery as different populations adapt to different environmental conditions.

Plasticity of photosynthetic mechanisms in *Eleocharis*

Although several other genera, such as *Panicum* (Edwards et al., 1982; Gutierrez et al., 1974; Hatch et al., 1975; Leonardos and Grodzinski, 2000; Ohsugi and Murata, 1986; Ohsugi et al., 1982) and *Flaveria* (Drincovich et al., 1998; Edwards and Ku, 1987; Ku et al., 1983; Monson et al., 1986) are also known to have diverse photosynthetic mechanisms, the unique feature of the genus *Eleocharis* is the genetic plasticity possessed by some individuals to modify their photosynthetic mechanism in adaptation to the environment, presumably to maximize photosynthetic carbon gain. Earlier studies by Ueno and colleagues (Agarie et al., 2002; Ueno et al., 1988, 1998, Ueno, 2004) demonstrated that *E. vivipara* type 1, is biochemically and anatomically C_4, but switches to C_3 photosynthesis fully when grown under submerged conditions (Ueno et al., 1998). In this study, we have shown that two other populations of *E. vivipara* have different responses to submerged growth : type 2 is C_4 terrestrially and switches to a C_3-C_4 intermediate when submerged while type 3 switches from C_3-like in terrestrial conditions to C_3 photosynthesis with induction of a putative bicarbonate transport system. Clearly, there is differentiation in photosynthetic plasticity among the populations in their adaptation to submerged conditions. It is likely that these *Eleocharis* species possess novel genetic mechanisms for regulating C_4 gene expression in response to environmental stimuli both among species and among different *E. vivipara* populations. The apparent acquisition of a bicarbonate transport system by *E. vivipara* type 3 while submerged may

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also represent an adaptation to low CO_2 conditions. Overexpression of a cyanobacteria gene involved in active uptake of bicarbonate in *Arabidopsis* and tobacco has led to enhanced photosynthesis and growth under limiting CO_2 conditions (Lieman-Hurwitz et al., 2003). Research into the use of bicarbonate ions in submerged or amphibious angiosperms is limited. Preliminary data suggests that *Myriophyllum spicatum* is capable of using bicarbonate under certain conditions (Bowes and Salvucci, 1989).

The switch in photosynthetic mode under submerged conditions may be related to the degree of C_4 characteristics (both biochemistry and anatomy) expressed in the terrestrial form. There is a clear trend in that expression of C_4 photosynthesis in a given species of *Eleocharis* tends to be attenuated in an aquatic environment. For example, values indicative of a typical C_4 (*E. vivipara* type 2) or C_4-like (*E. vivipara* type 1, *E. baldwinii*) plant become those of C_3-C_4 intermediates or C_3, respectively, whereas C_3-like (*E. vivipara* type 3) become C_3 when submerged. Previous studies have indicated that abscisic acid (ABA) may be involved in signaling the formation of C_4 characteristics, presumably due to water stress (Agarie et al., 2002; Ueno, 1998). Indeed, exogenous ABA caused submerged "C_3" plants (*E. vivipara* type 1) to develop Kranz anatomy in new culms and C_4-like biochemistry. Whether the expression of C_4 characteristics of submerged *E. vivipara* type 2 or 3 responds to ABA remains to be determined. Another aquatic plant, *Hydrilla verticillata*, has been shown to shift from C_3 to single-celled C_4 in response to high temperatures and long photoperiods resulting in decreased CO_2 in the aquatic environment (Magnin et al., 1997). It is speculated that the change in photosynthetic mechanisms in *Eleocharis* might actually be due to desiccation, thus resulting in the appearance of C_4 or C_4–like systems when the plants grow terrestrially

(Magnin et al., 1997). In additional studies with *Hydrilla verticillata*, along with *Elodea canadensis* and *Egeria densa*, application of ABA also induced the expression of PEPC and NADP-ME (Casati et al., 2000). Further study on the genetic mechanism and signaling pathway involved in this switch will aid our understanding of their novel adaptation to the environment.

Another interesting question is what would be the benefit of being C_4 in an environment where water is not limiting? The C_4 syndrome is believed to have evolved in hot, equatorial regions in response to limiting CO_2 conditions (Sage, 2001). The C_4 *Eleocharis* species studied grow in or near water where conditions would favor high stomatal conductance allowing for increased CO_2 diffusion into the photosynthetic culms. A hypothesis put forward by Ueno et al. (1989) suggests that it is a mechanism to overcome nitrogen deficiency. Waterlogged or flooded soils tend to be nutrient poor and C_4 plants have higher nitrogen use efficiency than C_3 plants, which may be the driving force for these plants, much as Venus flytraps ostensibly became carnivorous to increase their nitrogen intake. However, Robe and Griffiths (2000) believe that the carbon concentrating mechanisms (CCM) found in aquatic plants, such as in *Littorella uniflora*, which uses CAM when submerged, evolved in response to low CO_2 and high O_2 conditions found in certain aquatic habitats. *Littorella uniflora* apparently switches to C_3 when emergent with increased Rubisco activity (Beer et al., 1991). Thus plasticity between photosynthetic modes due to environmental fluctuations may be specific to families or even genera.

It is clear that *Eleocharis* species exhibit variation in carbon fixation pathways both within and among species. The adaptive significance of this variation is still unclear

and needs to be further explored. This study underlines the fact that there is variation among species and among populations within species; and, in this respect further studies are needed to understand the significance of this photosynthetic plasticity.

Materials and Methods

Plant growth conditions

All terrestrial *Eleocharis* plants (*E. erythropoda* Steudel, *E. baldwinii* (Torrey) Chapman, and *E. vivipara* Link) were grown in #1 nursery pots (approximately 1 L) filled with commercial potting soil and maintained in a greenhouse with a day/night temperature regime of 22-25°C/18-20°C. Sodium vapor lamps provided supplemental light, giving a minimum canopy PPFD of 120 μmol quanta m^{-2} s^{-1} for 12 hours. The plants were watered as needed and fertilized once a week. For submerged growth, established terrestrial plants were cut to within one inch of soil level and submerged into 38 L water tanks. Water level was maintained by addition of tap water as needed. The pH of the water ranged from 8.5 to 9.5. Plants were grown submerged for 1-2 months, generally developing 6-10 inches of new growth before analysis. Both submerged and terrestrial plants were grown at the same time and under the same conditions and newly matured culms produced under each condition were used for experiments.

Light microscopy

Samples (0.5 cm long) of terrestrial and submerged culms of all photosynthetic types were fixed overnight at 4°C in 2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde in 0.05 M PIPES buffer, pH 7.2. The samples were dehydrated with a graded ethanol series and embedded in London Resin White (Fort Washington, PA) acrylic resin. Cross sections, 1μm thick, were cut using a Reichert R Ultracut (Heidelberg, Germany) and dried from a drop of water onto gelatin-coated slides. Sections were stained with 1% (w/v) Toluidine Blue O in 1% (w/v) $Na_2B_4O_7$ for 30 s at room temperature and coverslipped with immersion oil. Slides were observed in an

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Olympus BH-2 microscope (Tokyo, Japan) coupled to a Microimage video system

(Boyertown, PA) and imaged with NIH Image 1.62 program (Bethesda, MD).

Determination of carbon isotope composition

Plant material was dried and ground to a fine powder. Subsamples (1.5-2.0 mg)

were analyzed by continuous flow isotope ratio mass spectrometry. A Eurovector

elemental analyzer was used to combust samples and separate the resulting carbon

dioxide gas, which was then analyzed for $^{13}C/^{12}C$ using a Micromass Isoprime mass

spectrometer. Results are expressed as $\delta^{13}C = 1000 \times (R_{sample} - R_{stamdard})/(R_{standard})$, where

$R_{standard}$ is Peedee belemnite (Craig, 1957). The precision based on replicate plant samples

was in the range of 0.1‰.

Enzyme assays and western immunoblot analysis

All enzyme assays were performed on crude extracts obtained by grinding tissue

harvested from the mid-portion of culms in the light in cold (-80°C) mortar and pestles

with sterile acid-washed sand and ice-cold grinding medium containing 50 mM HEPES

(or Tris) pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM Na-pyruvate, 10% Glycerol, 5%

insoluble-PVP, 5 mM iodoacetic acid, 10 mM $MgCl_2$, and 1% β-mercaptoethanol. The

crude extracts were cleared by centrifugation for 10 minutes at 14,000 x *g* at 4°C.

Enzymatic activity of phospho*enol*pyruvate carboxylase (PEPC), pyruvate

orthophosphate dikinase (PPDK), NADP-malic enzyme (ME), and NAD-ME were

assayed spectrophotometrically at 340 nm and 30°C by following the change in

NAD(P)H concentration. PEPC assay solution contained 50 mM Tris-HCl pH 8.0, 10

mM $NaHCO_3$, 5 mM $MgCl_2$, 0.25 mM NADH, 1 mM G-6-P, 4 mM PEP, 5 mM DTT,

and 12 units NAD-malate dehydrogenase in a 1 mL reaction mixture. Reaction was

initiated by the addition of plant soluble enzyme extract. PPDK assay solution contained

25 mM Hepes-KOH pH 8.0, 8 mM $MgSO_4$, 10 mM DTT, 10 mM $NaHCO_3$, 2 mM Na-

pyruvate, 5 mM $(NH_4)_2SO_4$, 1 mM G-6-P, 2.5 mM K_2HPO_4, 0.2 mM NADH, 0.5 units

PEPC and 2 units NAD-malate dehydrogenase. The reaction was initiated by the

addition of 20 μL of 50 mM ATP. Activity of NADP-ME was assayed in a premix of 25

mM Tris-HCl, pH 8.2, 1 mM EDTA, 20 mM $MgCl_2$, 0.5 mM NADP, and 5 mM malate

and initiated by addition of soluble enzyme extract. NAD-ME mix contained 25 mM

HEPES, pH 7.8, 0.25 mM EDTA, 2.5 mM DTT, 2.5 mM NAD, 5 mM malate, 8 mM

$(NH_4)_2SO_4$, 0.05 mM acetyl CoA, 0.075 mM CoA and the reaction was initiated with 50

μL of 120 mM MnCl.

Phospho*enol*pyruvate carboxykinase (PCK) assays were performed

spectrophotometrically at 280 nm and 30°C by measuring the change in oxaloacetate

(OAA) concentration in an assay solution containing 50 mM HEPES, pH 8.0, 2 mM

$MnCl_2$, and 0.3 mM OAA, pH 7.5 (made fresh daily). Immediately before assaying, 1

unit of pyruvate kinase was added and background activity was measured. Reactions

were initiated by the addition of 25 μL of 8 mM ATP, pH 7. Background activity was

subtracted from ATP-activated activity and compared to the absorbance of fresh OAA to

determine concentration and final enzyme activity.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was

measured using a premix of 0.5 M Tris-HCl, pH 8.0, 0.1 M $MgCl_2$, and 50 mM DTT.

Prior to assaying, 0.2 M $NaH^{14}CO_3$ and 20 uL extract were added and incubated at 30°C

for approximately 2 minutes. The reaction was initiated by adding 15 μL of 5.0 mM

RuBP and stopped after exactly 2 minutes by adding 50 μL of 1 N HCl/4 M formic acid

solution. Samples were air dried, resuspended in 50 μL dH$_2$O followed by 10 mL of scintillation cocktail and counted.

Chlorophyll content was determined using alcohol extracts of chlorophyll from enzyme extracts and measured at 665 and 649 nm according to Knudson et al. (1977).

For western immunoblotting, total soluble protein was extracted from culms by homogenizing tissue samples in extraction buffer. After centrifugation the supernatant was collected and protein concentration determined with Bio-Rad Bradford protein assay reagent using BSA as the standard. Protein samples (20 μg) were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit antisera raised against wheat Rubisco large subunit and maize PEPC. Goat anti-rabbit IgG conjugated to alkaline phosphatase was used to visualize the protein bands.

Photosynthetic gas exchange and O$_2$ inhibition

Terrestrial gas exchange was measured using the FastEst gas exchange system (FastEst, Tartu, Estonia), as described previously (Laisk and Oja, 1998) with an Applied Electrochemistry S-3A oxygen analyzer (Sunnyvale, CA) and Li-cor LI-6251 infra-red gas analyzer (Lincoln, NE). Assimilation rates, determination of Ci, light intensity (PPFD) and culm temperature were determined per Laisk and Loreto (1996). Photosynthetic rates for determination of oxygen inhibition were measured at 1000 μmol quanta m^{-2} s^{-1}, 360 μL L^{-1} CO$_2$, 80% relative humidity, 30°C, and at 2 and 21% O$_2$. Percent O$_2$ inhibition of photosynthesis was calculated as [(rate at 2% O$_2$ − rate at 21% O$_2$)/rate at 2% O$_2$] x 100. Conditions for determination of carboxylation efficiency and CO$_2$ compensation points were measured at 1000 μmol quanta m^{-2} s^{-1}, 80% relative humidity, 28°C, and 21% O$_2$.

Oxygen evolution rates of submerged plants

Oxygen evolution rates of submerged plants were measured using a Clark-type O_2 electrode at the base of a water-bath jacketed-Plexiglas chamber and voltage measured by a Hansatech CB1D (Hansatech, UK) connected to a chart recorder. Culms were cut approximately 0.25 cm long and incubated in 1 mL of 20 mM CO_2-free TRIS (pH 8.0) inside the electrode chamber. Buffer had previously been bubbled with air or 2% O_2 /98% N_2 gas mixture (for 21% and 2% O_2 measurements, respectively). CO_2-free buffer was prepared according to Levavasseur et al. (1997). Assays were conducted at 28ºC with temperature maintained by a water bath. Following dark respiration measurements, culms were illuminated under 500 μmol quanta m^{-2} s^{-1} PPFD using a Cole-Palmer Illuminator Model #9741-50 (Chicago, IL) and provided with 0.5 mM, 1.0 mM or 2.0 mM $NaHCO_3$ (pH 8.0) for free CO_2 concentrations of 11, 22, and 44 μM, respectively. Chlorophyll was quantified as described in Knudson et al. (1977).

Immunolocalization

Culm samples used for immunolocalization were prepared as described as above for anatomy. Cross sections were blocked for 1 h with TBST+BSA (10 mM Tris-HCl, 150 mM NaCl, 0.1% v/v Tween 20, 1% w/v bovine serum albumin, pH 7.2). The slides were then incubated for 3 h with either anti-Rubisco (1:500 dilution) or anti-PEPC (1:100 dilution) antibodies. Antibodies used (all raised in rabbit) were anti-spinach Rubisco (LSU) IgG (courtesy of B. McFadden, Washington State University) and commercially available anti-maize phosphoenolpyruvate carboxylase (PEPC) IgG (Chemicon, Temecula, CA). The slides were then washed with TBST+BSA and treated for 1 h with protein A-gold (10 nm particles diluted 1:100 with TBST + BSA). After washing, the

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sections were exposed to a silver enhancement reagent for 20 min according to the manufacturer's instructions (Amersham, Arlington Heights, IL), stained with 0.5% (w/v) Safrinin O, and imaged in a reflected/transmitted mode using a BioRad 1024 confocal system with Nikon Eclipse TE 300 inverted microscope (Hercules, CA) and Lasergraph image program 3.10.

Table legends:

Table I. Activities of key C_3 and C_4 enzymes (μmol mg chl^{-1} h^{-1}) and culm $\delta^{13}C$ (‰) values for *Eleocharis* species grown under either terrestrial or submerged conditions. Three representative C_4 species were grown terrestrially: *Zea mays* – NADP-malic enzyme (NADP-ME) type, *Amaranthus cruentus* – NAD-malic enzyme (NAD-ME) type and *Panicum texanum* – phospho*enol*pyruvate carboxykinase (PCK) type. Other enzymes measured were Ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco), phospho*enol*pyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK). Where standard errors are shown data are for 3 to 5 replicates. The data without standard errors for representative C_4 types (*Z. mays, A. cruentus, P. texanum*) are published values.

Table 1

Species	PEPC	PPDK	NADP-ME	NAD-ME	PCK	Rubisco	$\delta\ ^{13}C$
Z. mays (NADP-ME)	995	159	434	113+/-10	139+/-59	351+/-19	-12.5+/-0.2
A. cruentus (NAD-ME)	760	115	35	256+/-52	86+/-9	159+/-14	-13.9+/-0.2
P. texanum (PEP-CK)	2509	144	47	187+/-3	480+/-125	308+/-14	-12.0+/-0.3
E. erythropoda							
Terrestrial	124+/-16	28+/-2	12+/-1	14+/-1.6	nd	1110+/-27	-30.8+/-0.2
Submerged	68+/-18	19+/-3	22+/-3	15+/-0.9	nd	251+/-5	-30.5+/-0.2
E. vivipara type 3							
Terrestrial	905+/-65	139+/-37	7+/-1	128+/-29	nd	687+/-63	-16.7+/-0.1
Submerged	432+/-141	12+/-4	21+/-4	55+/-5.1	nd	372+/-41	-6.7+/-0.3
E. baldwinii							
Terrestrial	1719+/-165	152+/-7	6+/-1	301+/-40	nd	729+/-7	-15.0+/-0.2
Submerged	268+/-32	38+/-2	48+/-7	41+/-6	nd	277+/-18	-16.6+/-0.2
E. vivipara type 2							
Terrestrial	1947+/-114	125+/-5	15+/-2	265+/-37	nd	352+/-13	-13.2+/-0.1
Submerged	376+/-71	34+/-6	34+/-3	464+/-31	nd	135+/-5	-18.9+/-0.2
E. vivipara type 1[A, B]							
Terrestrial	1044+/-163	107+/-16		215+/-33		524+/-186	-15.4[B]
Submerged	259+/-69	10+/-3		34+/-10		673+/-179	-25.9[B]

[A]**Ueno et al., 1998**
[B]**Ueno et al., 1988**

Figure legends:

Figure 1. Light microscopy of *Eleocharis* species' culm anatomy grown under terrestrial or submerged conditions. Scale bar is 50 μm in length.

Figure 2. Western immunoblots for phospho*enol*pyruvate carboxylase (PEPC, 110 kD) and Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (Rubisco LSU, 56 kD) for *Eleocharis* species grown under terrestrial (T) or submerged (S) conditions and *Amaranthus cruentus* (NAD-ME type C_4) under terrestrial conditions.

Figure 3. Assimilation rate (A) and carboxylation efficiency (B) in terrestrial *Eleocharis* species. Photosynthetic measurements were made at 360 μL L^{-1}, 1000 μmol quanta m^{-2} s^{-1}, 80% relative humidity, 30°C and 21% or 2% O_2. Carboxylation efficiency was determined from the initial photosynthetic CO_2 response curve measured at 21% O_2. Error bars represent standard error of 2-3 replicates.

Figure 4. Oxygen inhibition of photosynthesis (A) and CO_2 compensation point (B) in terrestrial *Eleocharis* species. Photosynthetic measurements were made at 360 μL L^{-1}, 1000 μmol quanta m^{-2} s^{-1}, 80% relative humidity, 30°C and 21% or 2% O_2. The CO_2 compensation point was determined from the intercept of the initial photosynthetic CO_2 response curve measured at 21% O_2. Error bars represent standard error of 3-5 replicates.

Figure 5. Oxygen evolution rates of submerged *Eleocharis* species. Photosynthetic measurements were made at 500 μmol quanta m^{-2} s^{-1}, 28°C, 21% O_2 and varying

bicarbonate concentrations at pH 8.0. Bicarbonate concentrations were 0.5, 1.0, and 2.0 mM, which equates to 11, 22, and 44 μM free CO_2 in solution. Error bars represent standard error of 3 replicates.

Figure 6. Immunolocalization of Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phospho*enol*pyruvate carboxylase (PEPC) in culms of *Eleocharis* species grown under terrestrial conditions. Images A through D are Rubisco labeling, E through H are PEPC. Similar immunolocalization images are available for all submerged plants but are excluded here due to space constraints. Patterns of localization in the submerged forms are similar to that of terrestrially grown plants, but with a much lower labeling of the two enzymes. Scale bar is 50 μm in length.

Figure 1.

E. erythropoda | E. vivipara type 3 | E. baldwinii | E. vivipara type 2

Terrestrial

Submerged

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Figure 2.

Figure 3.

A

B

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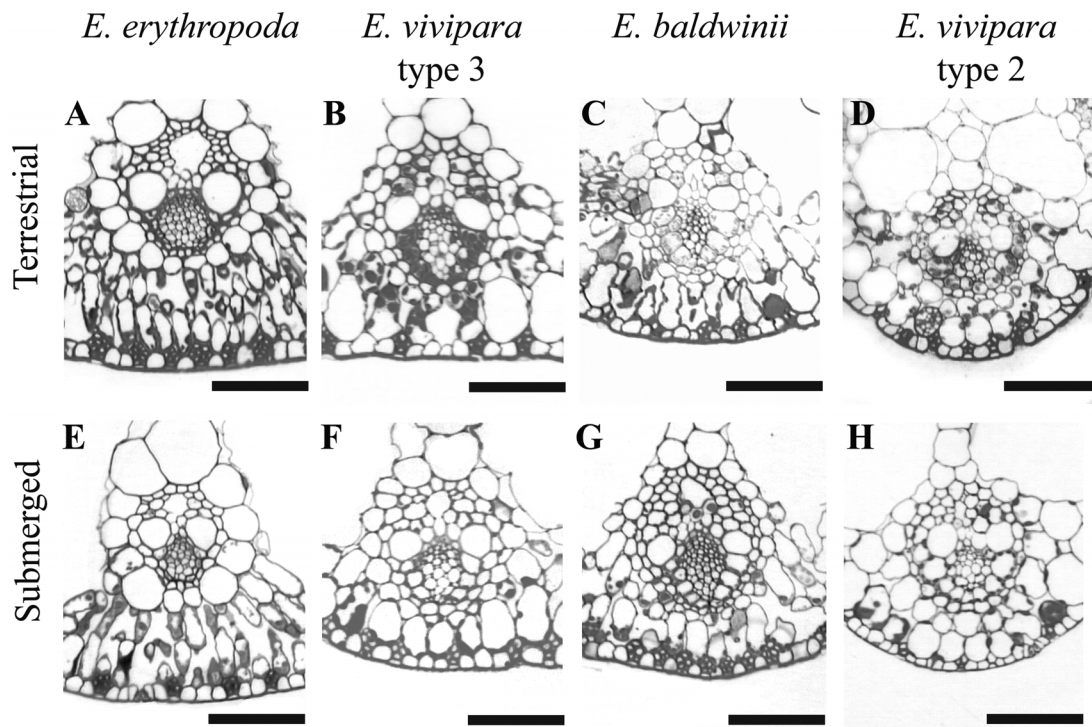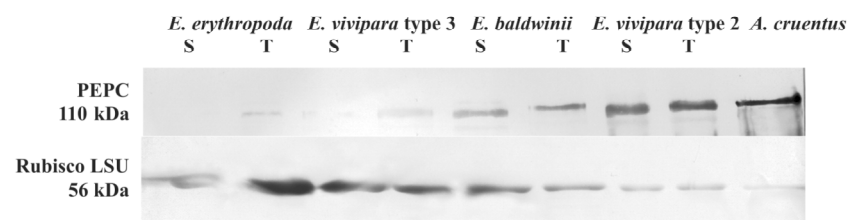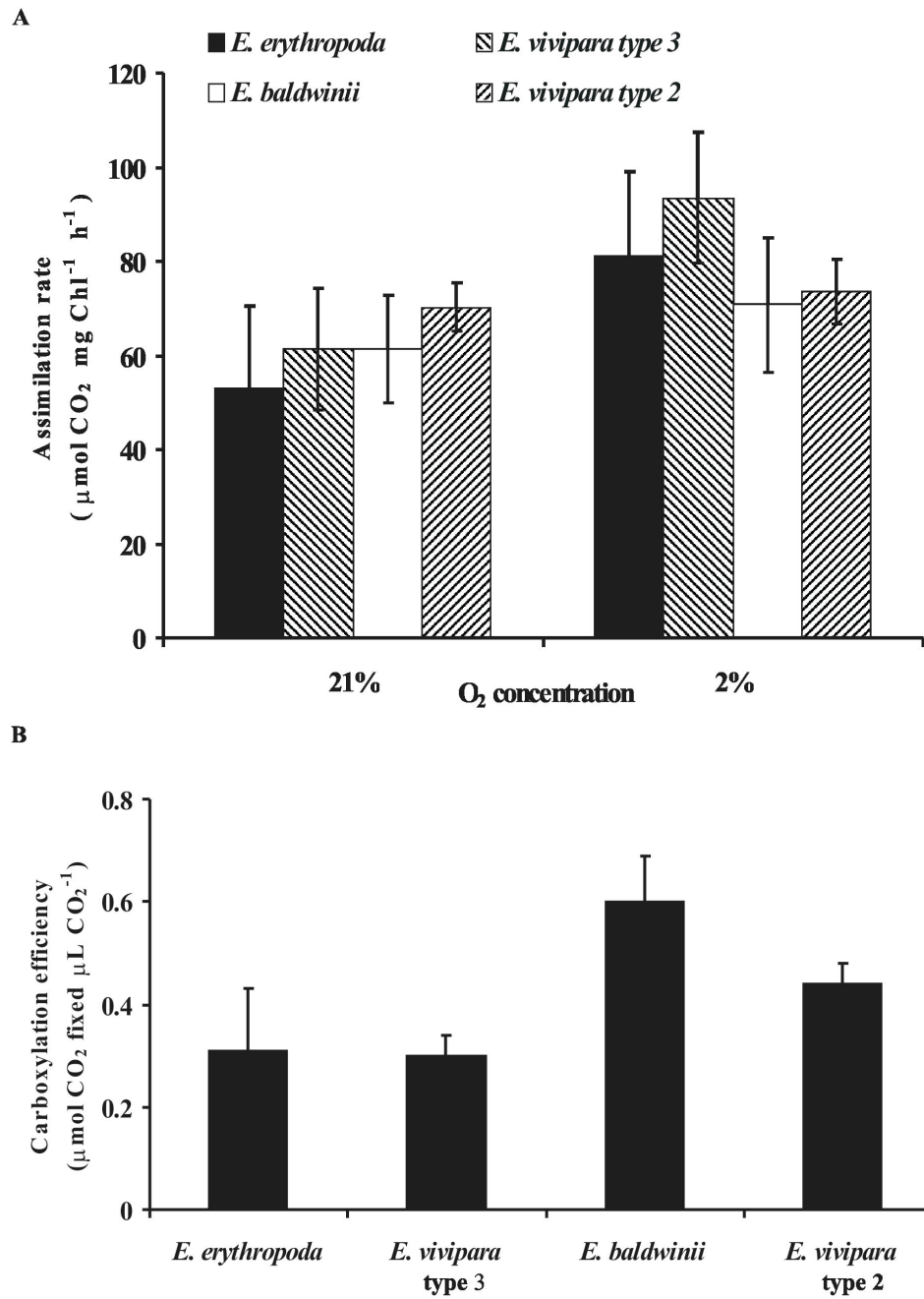
Figure 4.

Figure 5.

Figure 6.

| | *E. erythropoda* | *E. vivipara*
type 3 | *E. baldwinii* | *E. vivipara*
type 2 |

Figure 6.

E. erythropoda   E. vivipara type 3   E. baldwinii   E. vivipara type 2

Rubisco

PEPC
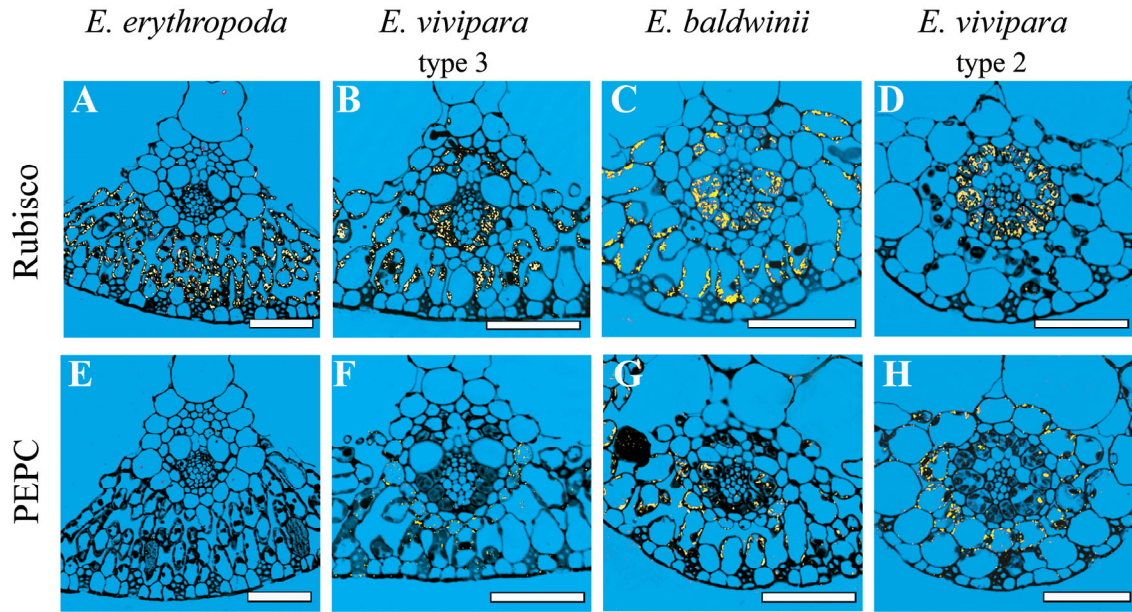
LITERATURE CITED

Agarie S, Kai M, Takatsuji H, Ueno O. (1997) Expression of C_3 and C_4 photosynthetic characteristics in the amphibious plant *Eleocharis vivipara*: structure and analysis of the expression of isogenes of pyruvate, orthophosphate dikinase. Plant Mol Bio 34: 363-369

Agarie S, Kai M, Takatsuji H, Ueno O (2002) Environmental and hormonal regulation of gene expression of C_4 photosynthetic enzymes in the amphibious sedge *Eleocharis vivipara*. Plant Sci 163: 571-580

Beer S, Sand-Jensen K, Vindbaek MT, Nielsen SL (1991) The carboxylase activity of Rubisco and the photosynthetic performance in aquatic plants. Oecologia 87: 429-434

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. Aquat Bot 34: 233-286

Bruhl JJ, Stone NE, Hattersley PW (1987) C_4 acid decarboxylation enzymes and anatomy in sedges (Cyperaceae): First record of NAD-malic enzymes species. Aust J Plant Physiol 14: 719-728

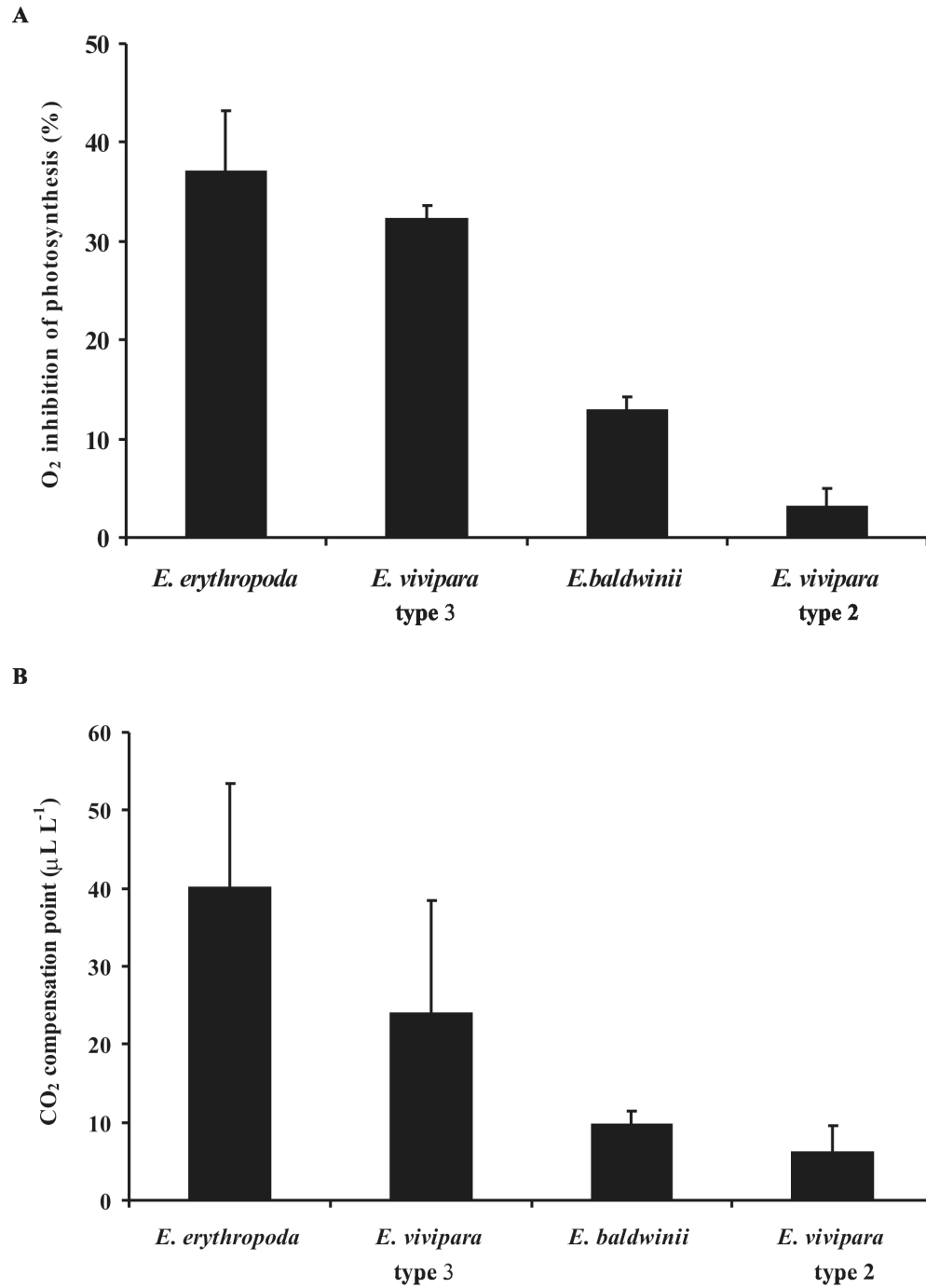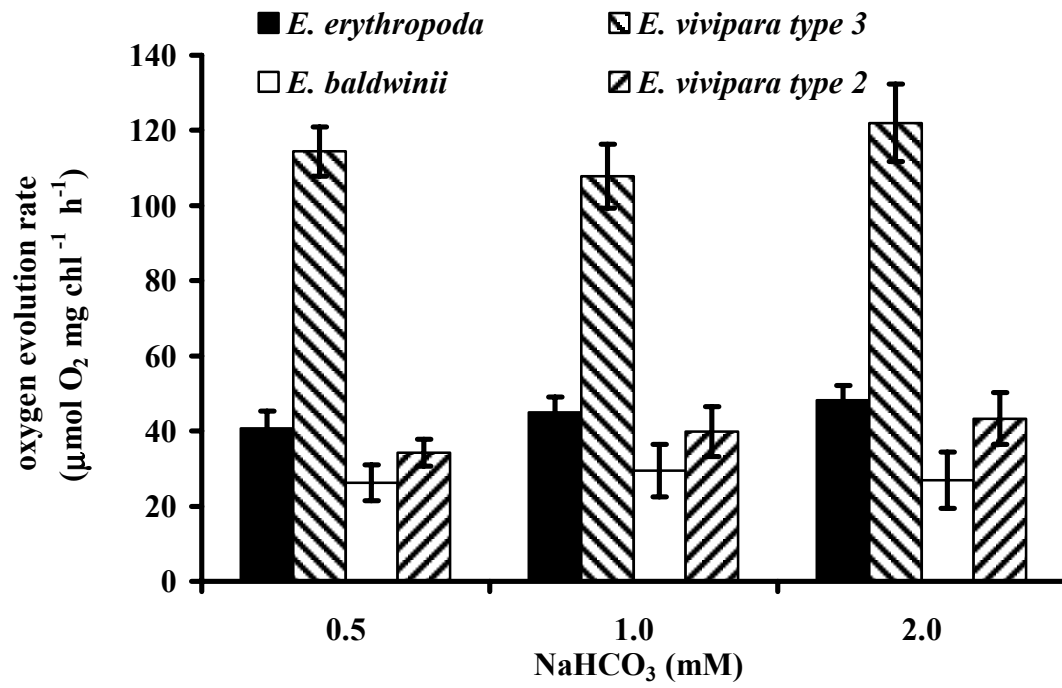Bruhl JJ (1995) Sedge genera of the world: relationships and a new classification of the Cyperaceae. Aust Syst Bot 8: 125-305

Bruhl JJ, Perry S (1995) Photosynthetic pathway-related ultrastructure of C_3, C_4 and C_3-like C_3-C_4 intermediate sedges (Cyperaceae), with special reference to *Eleocharis*. Aust J Plant Physiol 22: 521-530

Casati P, Lara MV, Andreo CS (2000) Induction of a C_4-like mechanism of CO_2 fixation in *Egeria densa*, a submersed aquatic species. Plant Physiol 123: 1611-1621

Cerling TE (1999) Paleorecords of C_4 plants and ecosystems. In: C_4 Plant Biology, RF

Sage and RK Monson, eds. Academic Press: New York. pp 445-469

Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-

spectrometric analysis of carbon dioxide. Geochim Cosmochim Acta 12: 133-149

Drincovich MF, Casati P, Andreo CS, Chessin SJ, Franceschi VR, Edwards GE, Ku MSB

(1998) Evolution of C_4 photosynthesis in *Flaveria* species. Plant Physiol 117:

733-744

Edwards GE, Ku MSB, Hatch MD (1982) Photosynthesis in *Panicum miliodes*, a species

with reduced photorespiration. Plant Cell Physiol 23: 1185-1195

Edwards GE, Ku MSB (1987) Biochemistry of C_3-C_4 intermediates. In: The

Biochemistry of Plants, PK Stumpf and EE Conn, eds, Vol. 10. Photosynthesis,

pp 275-325

González-Elizondo MS, Peterson PM (1997) A classification of and key to the

supraspecific taxa in *Eleocharis* (Cyperaceae). Taxon 46: 433-449

Gutierrez M, Gracen VE, Edwards GE (1974) Biochemical and cytological relationships

in C_4 plants. Planta 119: 279-300

Hatch MD, Kagawa T, Graig S (1975) Subdivision of C_4-pathway species based on C_4

acid decarboxylating systems and ultrastructural features. Aust J Plant Physiol 2:

111-128

Knudson LL, Tibbitts TW, Edwards GE (1977) Measurement of ozone injury by

determination of leaf chlorophyll concentration. Plant Physiol 60: 606-608

Ku MSB, Monson RK, Littlejohn RO, Nakamoto H, Fisher DB, Edwards GE (1983)

> Photosynthetic characteristics of C_3-C_4 intermediate *Flaveria* species. 1. Leaf
> anatomy, photosynthetic responses to O_2 and CO_2, and activities of key enzymes
> in the C_3 and C_4 pathways. Plant Physiol 71: 944-948

Ku MSB, Wu J, Dai Z, Scott RA, Chu C, Edwards GE (1991) Photosynthetic and

> photorespiratory characteristics of *Flaveria* species. Plant Physiol 96: 518-528

Laisk A, Loreto F (1996) Determining photosynthetic parameters from culm CO_2

> exchange and chlorophyll fluorescence. Plant Physiol 110: 903-912

Laisk A, Oja V (1998) Dynamics of culm photosynthesis. Commonwealth Scientific and

> Industrial Organization Publishing, Collingwood, Australia

Leonardos ED, Grodzinski B (2000) Photosynthesis, immediate export and carbon

> partitioning in source leaves of C_3, C_3-C_4 intermediate, and C_4 *Panicum* and
> *Flaveria* species at ambient and elevated CO_2 levels. Plant Cell Environ 23: 839-
> 851

Levavasseur G, Edwards GEE, Osmond CB, Ramus J (1991) Inorganic carbon limitation

> of photosynthesis in *Ulva rotundata* (Chlorophyta). J Phycol 27: 667-672

Lieman-Hurwitz J, Rachmilevitch S, Mittler R, Marcus Y, Kaplan A (2003) Enhanced

> photosynthesis and growth of transgenic plants that express *ictB*, a gene involved
> in HCO_3^- accumulation in cyanobacteria. Plant Biotechnol J 1: 43-50

Magnin NC, Cooley BA, Reiskind JB, Bowes G (1997) Regulation and localization of

> key enzymes during the induction of Kranz-less, C_4 -type photosynthesis in
> *Hydrilla verticillata.* Plant Physiol 115: 1681-1689

Monson RK, Moore BD, Ku MSB, Edwards GE (1986) Cofunction of C_3- and C_4-photosynthetic pathways in C_3, C_4, and C_3-C_4 intermediate *Flaveria* species. Planta 168: 493-502

Muasya AM, Simpson DA, Chase MW, Culham A (1998) An assessment of suprageneric phylogeny in Cyperaceae using *rbcL* DNA sequences. Plant Syst Evol 211: 257-271

Ohsugi R, Murata T, Chonan N (1982) C_4 syndrome of the species in the *Dichotomiflorum* group of the genus *Panicum* (Gramineae). Bot Mag Tokyo 595: 339-347

Ohsugi R, Murata T (1986) Variations in the leaf anatomy among some C_4 *Panicum* species. Ann Bot 58: 443-453

Roalson EH, Friar EA (2000) Infrageneric classification of *Eleocharis* (Cyperaceae) revisited: evidence from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Syst Bot 25: 323-336

Robe WE, Griffiths H (2000) Physiological and photosynthetic plasticity in the amphibious, freshwater plant, *Littorella uniflora*, during the transition from aquatic to dry terrestrial environments. Plant Cell Environ 23: 1041-1054

Sage RF (2001) Environmental and evolutionary preconditions for the origin and diversification of the C_4 photosynthetic syndrome. Plant Biol 3: 202-213

Sage RF (2004) The evolution of C_4 photosynthesis. New Phytol 161: 341-370

Soros CL, Dengler NG (2001) Ontogenetic derivation and cell differentiation in photosynthetic tissues of C_3 and C_4 Cyperaceae. Am J Bot 88(6): 992-1005

Takeda T, Ueno O, Samejima M, Ohtani T (1985) An investigation for the occurrence of
C$_4$ photosynthesis in the Cyperaceae from Australia. Bot Mag Tokyo 98: 393-411

Uchino A, Samejima M, Ishii R, Ueno O (1995) Photosynthetic carbon metabolism in an
amphibious sedge, *Eleocharis baldwinii* (Torr.) Chapman: modified expression of
C$_4$ characteristics under submerged aquatic conditions. Plant Cell Physiol 36:
229-238

Uchino A, Sentoku N, Nemoto K, Ishii R, Samejima M, Matsuoka M (1998) C$_4$-type
gene expression is not directly dependent on Kranz anatomy in an amphibious
sedge *Eleocharis vivipara* Link. Plant J 14: 565-572

Ueno O, Takeda T, Murata T (1986) C$_4$ acid decarboxylating enzyme activities of C$_4$
species possessing different Kranz anatomical types in the Cyperaceae.
Photosynthetica 20: 111-116

Ueno O, Samejima M, Muto S, Miyachi S (1988) Photosynthetic characteristics of an
amphibious plant, *Eleocharis vivipara*: Expression of C$_4$ and C$_3$ modes in
contrasting environments. Proc Natl Acad Sci 85: 6733-6737

Ueno O, Samejima M (1989) Structural features of NAD-malic enzyme type C$_4$
Eleocharis: an additional report of C$_4$ acid decarboxylation types of the
Cyperaceae. Bot Mag Tokyo 102: 393-402

Ueno O, Samejima M, Koyama T (1989) Distribution and evolution of C$_4$ syndrome in
Eleocharis, a sedge group inhabiting wet and aquatic environments, based on
culm anatomy and carbon isotope ratios. Ann Bot 64: 425-438

Ueno O (1996a) Immunocytochemical localization of enzymes involved in the C_3 and C_4

    pathways in the photosynthetic cells of an amphibious sedge, *Eleocharis vivipara*.

    Planta 199: 394-403

Ueno O (1996b) Structural characterization of photosynthetic cells in an amphibious

    sedge, *Eleocharis vivipara*, in relation to C_3 and C_4 metabolism. Planta 199: 382-

    393

Ueno O (1998) Induction of Kranz anatomy and C_4-like biochemical characteristics in a

    submerged amphibious plant by abscisic acid. Plant Cell 10: 571-583

Ueno O (2001) Environmental regulation of C_3 and C_4 differentiation in the amphibious

    sedge *Eleocharis vivipara*. Plant Physiol 127: 1524-1532

Ueno O (2004) Environmental regulation of photosynthetic metabolism in the

    amphibious sedge *Eleocharis baldwinii* and comparisons with related species.

    Plant Cell Environ 27: 627-639

Ueno O, Wakayama M (2004) Cellular expression of C_3 and C_4 photosynthetic enzymes

    in the amphibious sedge *Eleocharis retroflexa* ssp. *chaetaria*. J Plant Res 117:

    433-441

CONCLUSION

It is predicted that by 2050, the world's population could increase by a third (Surridge, 2002) and as the world's population continues to grow it is imperative that additional food supplies are provided. To accomplish this goal without further destruction to the environment, increased land usage or fertilization, creative research is needed. One approach is to engineer C_4 photosynthesis into crops, such as rice, which grows in warm climates where C_4 is of benefit. Using the single-cell model of C_4 photosynthesis may provide insight into the possibility of "Tuning up crop photosynthesis" (Edwards, 1999). With further research showing that "Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis" (Voznesenskaya et al., 2001) the idea of genetically engineering a C_4 plant is no longer so far fetched. With climate change, rising levels of CO_2 may partly negate the value of C_4 rice, but concomitant rises in temperature and drought could increase the benefit of C_4. Based on ratios of parameters in maize to rice, if rice was made C_4 there would be increases in water use efficiency of 89%, photosynthetic nitrogen use efficiency of 180%, and radiation use efficiency of 50% (Mitchell and Sheehy, 2006). Additionally, the projected increase in atmospheric CO_2 in the next 50 years will still not be at levels to saturate Rubisco and negate oxygen inhibition (Bowes et al., 2003). Moreover, the potential for increased tolerance of these plants to oxidative and drought stress, stress from heavy metals (namely aluminum) and potential for greater nutrient uptake, could make them even more valuable with increased salinization of agricultural lands and other abiotic stresses.

To that end, the transformation of rice with genes encoding PEPC and PPDK, as described in this paper, shows that the insertion of one or two C_4 genes causes some

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changes in the physiology of these plants, which may be beneficial. Both PC and CK rice showed decreased CO_2 compensation points at 21 and 2% O_2, higher light saturated rates of photosynthesis under atmospheric conditions, higher stomatal conductance at 2% O_2, and increases in Ci/Ca ratios when compared to wild-type rice, though only PC rice showed a change in carbon isotope ratios. PC rice had the same optimum photosynthetic temperature as wild-type rice, but had higher photosynthetic rates at higher temperatures. CK rice had a lower optimum temperature than wild-type (30, compared to 35 °C), and had higher photosynthetic rates from 15 to 35°C, but lower rate at 40°C. All three, PC, CK, and wild-type, rice lines had equivalent Rubisco activity. The proposed theory that PC rice has higher stomatal conductance due to the synthesis of malate in stomata (Ku et al., 1999) would explain several observations in this study: higher Ci/Ca ratio, more negative $\delta^{13}C$ value, and increased photosynthetic rates. This was supported in the current study showing higher stomatal conductance in PC and CD than wild-type under 2% O_2, although not under 21% O_2. It would be of value to look at these parameters across a range of transformants, and see what, if any values, may show differences based on levels of expression of PEPC and PPDK enzymes. Previously, different PC lines of rice with varying PEPC activity levels were analyzed. O_2 inhibition and $\delta^{13}C$ values decreased with increasing PEPC activity and up to a certain level of PEPC activity, photosynthetic rates also increased until a limit was reached that resulted in lower photosynthesis and decreased chlorophyll levels (Ku et al., 2000). Based on preliminary photosynthetic data in that study, there appeared to be some correlation between stomatal conductance, internal CO_2 concentration, and PEPC; however, it would be interesting to make a rigorous statistical analysis of PC and CK lines, comparing enzyme activity,

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stomatal conductance and Ci/Ca ratios. A comparison across transgenic PC lines, with a range of PEPC activity from ten to 100-fold higher activity than wild-type, showed an apparent decrease in oxygen insensitivity with increasing PEPC amounts, but this was due to a decrease in photosynthetic stimulation by lower oxygen (Agarie et al., 2002). In the current study, PC rice showed a decrease in oxygen sensitivity, but had a higher photosynthetic rate at 2% oxygen compared to wild-type rice at all CO_2 levels measured. Unfortunately, it is almost impossible to make comparison to published data by other researchers due to the use of a single transformant in the majority of studies. Because of the variability of responses based on the amount of activity/protein present in the lines, currently no ultimate conclusion can be drawn. For PPDK transformants, only preliminary genetic analyses have been done, and no studies have been published on CK rice other than the preliminary data of Jiao et al. (2002). However, the CK rice was developed from homozygous lines of the PC and PK rice, which were also used in the aforementioned study. Future studies could benefit by different research groups including a common set of transformants in both laboratory and field studies.

Additional research, such as that with *Eleocharis* (Chapter 4), not only illustrates the diversity and plasticity of the genus, but also may provide some insight into the mechanisms of C_4 photosynthesis. While the biochemistry and genes encoding the essential enzymes have been well documented, the genes encoding the anatomical specialization of Kranz anatomy, and the inducibility of C_4 in *Eleocharis*, are unknown. If these could be determined, the ability to install the C_4 pathway into C_3 plants may be more achievable. Studies with *Eleocharis*, combined with those of *Hydrilla*, *Elodea* and other aquatic facultative C_4 species may enable such a discovery. Alternatively, as

proposed by Brown et al. (2005), use of the genus *Cleome*, with its C_3 and C_4 species, and its close relation to *Arabidopsis*, could provide a vast wealth of information due to the availability of genetic tools developed for use with *Arabidopsis*, in addition to the benefits of short life cycles, relatively small genome and small plant size of some C_4 *Cleome* species.

LITERATURE CITED

Agarie S, Miura A, Sumikura R, Tsukamoto S, Nose A, Arima S, Matsuoka M, Miyao-Tokutomi M (2002) Overexpression of C4 PEPC caused O_2-insensitive photosynthesis in transgenic rice plants. Plant Sci 162: 257-265

Bowes G, Rao SK, Reiskind JB (2003) Photosynthetic acclimation of rice to global climate change: Will a same-cell C_4 system help? In TW Mew, DS Brar, S Peng, D Dawe, B Hardy, eds, Rice science: innovations and impact for livelihood. Proceedings of the International Rice Research Conference, 16-19 September 2002, Bejing, China. Bejing (China): International Rice Research Institute, Chinese Academy of Engineering, and Chinese Academy of Agricultural Sciences.

Brown NJ, Parsley K, Hibberd JM (2005) The future of C_4 research-maize, *Flaveria* or *Cleome*? Trends Plant Sci 10(5): 215-221

Edwards G (1999) Tuning up crop photosynthesis. Nat Biotechnol 17: 22-23

Jiao D, Huang X, Li X, Chi W, Kuang T, Zhang Q, Ku MSB, Cho D (2002) Photosynthetic characteristics and tolerance to photo-oxidation of transgenic rice expressing C_4 photosynthetic enzymes. Photosynth Res 72: 85-93

Ku MSB, Agarie S, Nomura M, Fukayama H, Tsuchida H, Ono K, Hirose S, Toki S, Miyao M, Matsuoka M (1999) High-level expression of maize phospho*enol*pyruvate carboxylase in transgenic rice plants. Nat Biotechnol 17: 76-80

Ku MSB, Cho D, Ranade U, Hsu TP, Li X, Jiao DM, Ehleringer J, Miyao M, Matsuoka M (2000) Photosynthetic performance of transgenic rice plants overexpressing

maize C_4 photosynthesis enzymes. In JE Sheehy, PL Mitchell, B Hardy, eds, Redesigning rice photosynthesis to increase yield. Proceedings of the workshop on the quest to reduce hunger: redesigning rice photosynthesis. Elsevier Science B.V., IRRI and Amsterdam, pp 193-204

Mitchell PL, Sheehy JE (2006) Supercharging rice photosynthesis to increase yield. New Phytol 171: 688-693

Surridge C (2002) The rice squad. Nature 416: 576-578

von Caemmerer S (2003) C_4 photosynthesis in a single C_3 cell is theoretically inefficient but may ameliorate internal CO_2 diffusion limitations in C_3 leaves. Plant Cell Environ 26: 1191-1197

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414: 543-546